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Increased frequency of CD4+PD-1+HLA-DR+ T cells is associated with disease progression in CLL

Lauren Elston¹, Chris Fegan¹, Robert Hills¹,#, Shaikh Shimaz Hashimdeen¹, Elisabeth Walsby¹, Peter Henley¹, Chris Pepper¹,² and Stephen Man¹,*

¹Division of Cancer and Genetics, Cardiff University School of Medicine, Cardiff CF14 4XN
²Brighton and Sussex Medical School, University of Sussex, Brighton, BN1 9PX

# Present address: Clinical Trial Service Unit & Epidemiological Studies Unit, Nuffield Department of Population Health, University of Oxford, Richard Doll Building, Oxford OX3 7LF

*Correspondence: Dr. Stephen Man
mans@cardiff.ac.uk

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Abstract

CLL patients often have abnormal expansions of CD4+ and CD8+ T cells and this can be associated with progressive disease. To characterize the key T cell populations involved in this phenomenon, we used flow cytometry and 11 phenotypic markers to study 74 CLL patients and 14 controls. T cells of CLL patients were more phenotypically complex than those of healthy controls with significant increases in the frequencies of CD4 and CD8 memory T cells expressing exhaustion, activation and senescence associated markers. Multivariate analysis of 111 different T cell subsets showed that high frequencies of 4 subsets (3 CD8 and 1 CD4) were associated with shorter progression free survival. The most significant association was with CD4+HLA-DR+PD-1+ T cells, and patients could be stratified into high and low risk groups based on the frequency of these T cells. The expansion of this CD4+ subset could not be accounted for by age, CMV infection or increases in Treg cells. Overall, these results highlight two relatively simple biomarkers, %CD8+ and %CD4+PD-1+HLA-DR+ T cells, which can be used to risk-stratify CLL patients, independent of other tumour-associated markers. They also provide further evidence for the pivotal role of T cells in modulating the pathology of CLL.
Introduction

It has been long recognised that CLL patients have abnormal CD4 and CD8 T cell phenotypes, including inversion of the CD4:CD8 ratio (Matutes et al., 1981; Mills & Cawley, 1982; Platsoucas et al., 1982; Mittelman et al., 1984) and accumulation of memory T cells with concomitant loss of naive T cells (Totterman et al., 1989; Serrano et al., 1997; Peller & Kaufman, 1991). These phenotypes resemble those associated with ageing (Olsson et al., 2000), and may be associated with CMV infection (Mackus et al., 2003; Pourgheysari et al., 2010). However, previous studies have shown that the T cell abnormalities in CLL are not simply due to CMV infection (Riches et al., 2013; Raa et al., 2014) or the natural ageing process (Nunes et al., 2012). This suggests a distinct, CLL-related effect in shaping the differentiation of T cell populations, but how this is achieved is unclear.

In a previous study of patients with early stage disease, we found that patients with abnormal expansions of CD8$^+$ T cells, and hence an inverted CD4:CD8 T cell ratio, had a shorter time to first treatment and a reduced progression-free survival (PFS) (Nunes et al., 2012). The expanded T cells in CLL frequently expressed markers associated with exhaustion (PD-1), findings also confirmed by others (Brusa et al., 2013; Riches et al., 2013; Tonino et al., 2012). However, human T cell subsets are complex and the expression of PD-1 may not necessarily define T cells that are functionally exhausted (Duraiswamy et al., 2011). To address some of these issues, and to confirm our earlier findings, we used polychromatic flow cytometry on a new CLL patient cohort to more precisely define the T cell populations that were expanded in CLL and to determine whether these phenotypic subsets were associated with disease progression.
Materials and Methods

Antibodies

The bulk of the analysis of the CLL patient and healthy donor samples was undertaken using two overlapping antibody panels designed to characterise human T cells subsets in combination with markers associated with activation, senescence and exhaustion. Further experiments on smaller groups of CLL patients were performed to investigate cytotoxic (GZMB), proliferating cells (MKI67) and Tregs. Full details of all antibody panels can be found in Table S1. Treg cell frequency was determined using a Treg kit (eBioscience) according to manufacturer’s instructions.

Flow Cytometry

Direct immunofluorescent staining was carried out as previously described (Nunes et al, 2012) before acquisition of samples on a BD FACSCanto II Cytometer (BD). Compensations were calculated and applied automatically by using an anti-Mouse Ig/negative control compensation particles set (BD) and FACSDiva software. Fluorochrome-conjugated monoclonal antibodies (at the same concentration added to the cell samples) were used as single-colour compensation controls. Gating of populations positive for any particular marker was based on fluorescence minus one (FMO) controls, followed by Boolean gating to determine the frequency of T cells expressing multiple markers. The gating scheme is shown in Fig S1. All analysis was performed using FlowJo software (versions 9-10.42).

Analysis and Statistics

The Mann-Whitney U test was used to analyses differences between two independent parameters while the Kruskal-Wallis test, with post-hoc Dunn’s correction, was applied for comparisons between three or more variables. Correlation analyses were performed using Spearman’s rank correlation coefficient and Kaplan-Meier curves were used to compare PFS and overall survival (OS) between categorical groups. For associations between CLLIR and clinical data, Fisher’s exact test was used. All univariate analyses were carried out using
PRISM 5.0 software (Graphpad Software). For analysis of T cell subsets SPICE (v5.3) software (Roederer et al, 2011) was used to create pie chart overviews and calculate significance between pie chart variables. Multivariate analysis of T cell populations associated with PFS and OS were performed using a Cox proportional hazards regression model with forward selection (Hyatt et al, 2017). P<0.05 was considered significant. Recursive partitioning was performed using values for % CD4⁺PD-1⁺HLA-DR⁺ and CD8⁺ from individual patients. These values were tested in a forward selection regression model and ranked according to hazard ratio and probability.

**Patient and control group blood samples**

CLL blood samples were obtained from 74 patients attending outpatient’s clinics at University Hospital of Wales and Llandough Hospital (age range 42-92). These were taken with informed consent and in accordance with the ethical approval granted by South East Wales Research Ethics Committee (13/WA/0346). A total of 63 patients were diagnosed as Binet Stage A, 7 as Stage B and 4 as Stage C. The median follow-up of the cohort (from diagnosis to sample date) was 6.2 (1-28) years. The cohort included patients (n=21) who had been previously treated (average of 5 years before sample, range 1-16 years). At the time of sample, the majority of these (20/21) were not undergoing active treatment, therefore all patients (n=74) were included in the statistical analysis. Clinical details of patients are shown in Table S2. Samples used as comparator healthy controls were obtained from healthy donors with informed consent (age range 46-84). Peripheral blood mononuclear cells (PBMC) were isolated via density gradient centrifugation using Ficoll-Histopaque (Histopaque-1077, Sigma) as previously described (Nunes et al, 2012). All flow cytometry was carried out on freshly isolated cells (between 6-12 hours post collection).
CMV serology

CMV (IgG) serostatus was determined from plasma samples in a blinded fashion by the Public Health Wales Microbiology Service in a subset of the patient cohort (n=63); 23 inverted ratio samples (CLLIR) and 40 normal ratio samples (CLLN).

Results

Inverted CD4:CD8 ratio is associated with inferior prognosis in CLL

We had previously reported a significant association between an inverted CD4:CD8 ratio and shorter PFS (Nunes et al, 2012). Follow-up of the 63 available patients from this cohort (7 years later) also demonstrated a significant association between inverted ratio and overall survival (OS) (Fig 1A). This result prompted us to validate our original findings on a new cohort of CLL patients and to perform a more detailed phenotypic analysis of the T cell subsets that were preferentially expanded in the inverted ratio patients.

A total of 74 new patients (Stage A, n=63; Stage B, n=7, Stage C, n=4) were studied with 36% (27/74) demonstrating an inverted CD4:CD8 ratio (<1.0). As in the previous study (Nunes et al, 2012), stratifying the patients into two groups based on an inverted ratio (<1.0, CLLIR) and a normal ratio (>1.0, CLLN), demonstrated a shorter PFS for the CLLIR patients (p=0.0002, Fig 1b). The inverted ratio appeared to be independent of other clinical markers such as disease stage (the majority of patients were stage A), lymphocyte doubling time (LDT), CD38 expression or IGHV mutational status (Fig S2A-D). Further, there was no association between inverted CD4:CD8 ratio and CMV serostatus or age (Fig S2E,F).

Phenotypic complexity of T cell subsets in CLL

Our previous study demonstrated the abnormal distribution of T cell subsets in early stage CLL patients. This was confirmed in the new cohort, with a relative expansion in the frequency of memory CD4+ and CD8+ T cells and a reduction in naive T cells detected by flow cytometry (Fig S3). We then performed detailed phenotypic analysis of T cell subsets
using two antibody panels that detected cell surface markers associated with exhaustion (PD-1) or senescence (KLRG-1).

Using antibody panel 1 and analysis with SPICE software (Roederer et al, 2011), there was an overall increase in PD-1$^+$ T cells in CLL compared to healthy donors (yellow arcs in Fig 2). These were a mixture of at least 4 sub-populations with frequent co-expression of the differentiation marker CD57 (red arcs) and the activation marker HLA-DR (light blue arcs). This was evident for both CD4$^+$ and CD8$^+$ T cells (Fig2A and 2B, Panel 1).

Previous studies had suggested that CLL patients had increased frequencies of T cells with a senescent phenotype compared to healthy controls (Nunes et al, 2012; Göthert et al, 2013). We confirmed these findings using an antibody panel that included markers (KLRG-1, CD57 and CD127) associated with terminal differentiation and senescence in T cells (Ibegbu et al, 2005). There was considerable phenotypic complexity in the expanded population of CD8$^+$KLRG-1$^+$ T cells in CLL, with at least 8 different sub-populations defined on co-expression of different cell surface markers (Fig 2a panel 2). This phenotypic complexity was also evident for CD4$^+$KLRG-1$^+$ T cells (Fig 2B, Panel 2).

Overall the SPICE analysis demonstrated the considerable complexity of the T cell pool in both the CD4$^+$ and CD8$^+$ T cell subsets, with many discrete sub-populations defined by co-expression of phenotypic markers. These T cell subsets could be further characterized on the basis of differentiation stage (naïve or memory). This made it challenging to determine the prognostic value of any given T cell population using our previous univariate approach (Nunes et al, 2012). Therefore, a combined dataset of 111 variables based on T cell subsets defined by the two antibody panels was generated for multivariate analysis (Table S3).

**Multivariate analysis defines T cell subsets associated with poorer prognosis**

We have shown in two separate cohorts of early stage CLL patients that preferential expansion of CD8$^+$ T cells are associated with inferior clinical prognosis (PFS in (Nunes et al, 2012) and PFS and OS in Fig 1A,B). In an attempt to precisely define the T cell subset
associated with this phenomenon, Cox-proportional hazards regression modelling with forward selection was used to test which T cell phenotypes (111 variables) had the strongest effect on PFS. Only four phenotypically defined T cell subsets were significantly associated with PFS at the P<0.05 level (Table S4). Three of these were CD8+, and included total %CD8 (p=0.018), %CD8+CD57+HLA-DR+ (p=0.029) and CD8+EMRA CD57+HLA-DR+PD-1+ (p=0.0218). However, the T cell population that demonstrated the most significant association with PFS was CD4+PD-1+HLA-DR+ (p<0.0001). This CD4 subset occurred at low frequency (<7%) in our cohort of healthy donors, but frequencies of up to 50% of CD4+ T cells were observed in CLL patients (Fig 3A,B), with the highest frequencies occurring in the effector memory (EM) subset (Fig 3A). The frequency of this subset was also negatively correlated with the CD4:CD8 ratio (Fig S4A) and positively correlated with %CD8 T cells (Fig S4B).

**Stratification of patient cohorts based on T cell phenotypes**

To further explore the association between the identified T cell subsets and clinical prognosis, we performed recursive partitioning, focusing on the two T cell subsets best associated with PFS; CD4+PD-1+HLA-DR+ and CD8+. For the latter subset a significant difference in PFS was demonstrated using the median value of 33.2% of CD8+ T cells to categorise CLL patients (Fig 4A). However the greatest reduction in PFS (and increase in hazard ratio) was observed using a %CD8 threshold of 56.9%; patients exceeding this threshold had more aggressive disease (Fig 4B). These results are consistent with our previous findings that CD8+ T cell expansion can be used as a biomarker for CLL patients with poorer prognosis and that changes in the proportions of T cell subsets may be a marker of advancing disease.

For CD4+PD-1+HLA-DR+ T cells, use of the median value (9.1%) as a cut-off divided the CLL patient cohort into two groups with significantly different PFS (hazard ratio = 3.78, p=0.0019, Fig 5A). Recursive partitioning of the %CD4+PD-1+HLA-DR+ T cells relative to
PFS, allowing greater discrimination between patient groups. For example, patients with

\[ >14.4\% \text{CD4}^+\text{PD-1}^-\text{HLA-DR}^+ \] T cells had poorer prognosis as measured by PFS (hazard ratio = 5.459, p<0.0001, Fig 5B.). Decreasing or increasing the threshold according to

%CD4^+PD-1^-HLA-DR^+ T cells had dramatic effects in this cohort. Patients with high % e.g.

31.5% CD4^+PD-1^-HLA-DR^+ T cells appeared to have more progressive disease within the

first 10 years (Fig 5C). In contrast, patients with a low % of CD4^+PD-1^-HLA-DR^+ T cells e.g. below 4.95% showed no sign of progression (Fig 5D). These initial results need to be

validated with larger patient numbers, particularly with regard to setting thresholds, but

nevertheless illustrate the potential for this biomarker to identify patient groups with different

rates of disease progression.

**CD4^+PD-1^-HLA-DR^+ T cells are not wholly Treg and their expansion is not associated with CMV serostatus or age**

CMV can cause expansions of the CD8^+ and CD4^+ T cell subsets in CLL (Mackus et al, 2003; Pourgheysari et al, 2010), but this has little clinical impact in terms of disease outcome (Parry et al, 2016). It was possible that the expanded CD4^+ T cell populations we observed, simply reflected a response to chronic CMV infection. We tested a subset of CLL patients (n=31) for CMV serostatus but no significant association was seen between the frequencies of CD4^+PD-1^-HLA-DR^+ T cells and CMV serostatus; relatively high frequencies of CD4^+PD-1^-HLA-DR^+ were detected in the CMV seronegative patient samples, and conversely low frequencies were detected in CMV seropositive samples (Fig 6A). T cell expansions can also occur as a consequence of age (Olsson et al, 2000). However, no correlation between %CD4^+PD-1^-HLA-DR^+ and patient ages was observed in this cohort (Fig S5C). Furthermore no associations were seen between %CD4^+PD-1+HLA-DR+ and other tumour associated markers such as CD38, IGHV status, stage, or LDT (Figure S6).

CD4^+ Treg cells have been reported to occur at increased frequency in CLL patients (D’Arena et al, 2011; Beyer et al, 2005). Our previous study demonstrated increased frequency of Treg
cells in CLL patient samples compared to age-matched controls, but this did not correlate with either inverted CD4:CD8 ratios or clinical prognosis (Nunes et al, 2012). Here we confirm that CLL patient samples contain increased frequencies of Treg cells when compared to age-matched controls. However, the relative expansion in Tregs would not be able to account for the greater frequencies of CD4⁺PD-1⁺HLA-DR⁺ T cells in CLL patients (Fig 6B). There was no correlation between the frequencies of CD4⁺PD-1⁺HLA-DR⁺ T cells and Treg cells in CLL patients (data not shown). Preliminary assessment of CD4⁺PD-1⁺HLA-DR⁺ T cells using functional markers for cytotoxicity (granzyme B) (Porakishvili et al, 2001) and proliferation (Ki67) (Palma et al, 2017) demonstrated that the majority of this subset were not cytotoxic or actively proliferating (Fig S7). Overall these results suggest that CD4⁺PD-1⁺HLA-DR⁺ T cells are a functionally heterogeneous subset of T cells that expand in CLL patients with progressive disease.
Discussion

In this study, we confirmed our previous observation that early stage CLL patients with an inverted CD4:CD8 ratio (CLL\textsuperscript{IR}) have a poorer prognosis (Fig 1). The inverted ratio was not associated with patient age, CMV serostatus or any tumour-associated prognostic marker (Fig S1). In addition, we performed a detailed phenotypic analysis of CLL\textsuperscript{IR} patients, and explored whether any differentially expressed T cell subsets were associated with prognosis. While multiple discrete CD8\textsuperscript{+} and CD4\textsuperscript{+} T cell populations were expanded in CLL patients relative to controls, the T cells that were most significantly associated with shorter PFS were CD4\textsuperscript{+} T cells that co-expressed PD-1 and HLA-DR. These T cells constituted up to 50% of the CD4\textsuperscript{+} T cell compartment in CLL patients but were generally present at low frequencies (1-7%) in healthy donors. This is the first description of the CD4\textsuperscript{+}PD-1\textsuperscript{+}HLA-DR\textsuperscript{+} subset in CLL and recursive partitioning showed that high frequency of CD4\textsuperscript{+}PD-1\textsuperscript{+}HLA-DR\textsuperscript{+} T cells identified patients with more aggressive disease.

This study started with the aim of defining the CD8\textsuperscript{+} T cells that expanded in the inverted ratio patients and were associated with poorer prognosis. However, this analysis, using 13 different markers, revealed a complex mixture of memory T cell subsets, with frequent co-expression of markers associated with exhaustion (PD-1), activation (HLA-DR) and senescence (KLRG-1) in both CD8\textsuperscript{+} and CD4\textsuperscript{+} T cell subsets. Determining the precise role of these subsets in disease will require further study of function and antigen specificity.

There have been several reports of distinct CD4\textsuperscript{+} T cell subsets that are increased in CLL patients relative to healthy controls. These include Treg cells (D'Arena et al, 2011), Tfh (Ahearne et al, 2013), Th17 (Hus et al, 2013), cytotoxic T cells (Porakishvili et al, 2001; Lindqvist et al, 2011) and CMV specific T cells (Pourghaysari et al, 2010). In this study, neither Treg or cytotoxic T cell frequencies could entirely account for the CD4\textsuperscript{+}PD-1\textsuperscript{+}HLA-DR\textsuperscript{+} T cell population. To our knowledge, this distinct T cell phenotype has not been
previously reported in CLL or implicated in the pathology of other diseases. However, T cells with a similar phenotype (CD4⁺PD-1⁺HLA-DR⁺CD38⁺) have been described in HIV infected patients (Eller et al, 2011), where increased frequencies (>6% of CD4⁺ T cells) were associated with progression to AIDS (Eller et al, 2011). It remains unclear why this particular population of CD4⁺ T cells was associated with poor prognosis in our CLL cohort and was a stronger predictor than the CD8⁺ T cell populations that are commonly preferentially expanded in CLL. CD4⁺ T cells from CLL patients can support the growth of CLL cells in vitro (Catakovic et al, 2017; Os et al, 2013) and in vivo after adoptive transfer into murine models (Bagnara et al, 2011). Whether the same is true of the CD4⁺PD-1⁺HLA-DR⁺ cells described in the current study remains to be determined. Therefore, it remains an open question whether the CD4⁺PD-1⁺HLA-DR⁺ T cells are responding to, or responsible for increasing tumour load in CLL.

Several studies of aged healthy populations (80-90+) have shown that inverted CD4:CD8 ratio can develop with age (up to 16% of the aged population). This results from decreases in CD4⁺ T cell number, and an increase in highly differentiated CD8⁺ memory cells expressing activation markers (Olsson et al, 2000; Wikby et al, 2008). This is strongly associated with CMV seropositivity. In contrast, CLL patients often demonstrate expansion of both CD4⁺ and CD8⁺ memory T cell compartments, but with a preferential expansion of CD8⁺ T cells resulting in the inverted ratio. There was no link between CMV serostatus and the inverted ratio or the frequency of CD4⁺PD-1⁺HLA-DR⁺ T cells. This implies that for some CLL patients, disease-associated processes are driving the expansion and differentiation of CD8⁺ and CD4⁺ T cells to create a prematurely aged immune phenotype.

The increased proportion of highly differentiated T cells (and concomitant decrease in naïve T cells) in CLL has other consequences. Although CAR-T cell therapy was pioneered in CLL and has demonstrated complete responses in some patients, the overall complete response
rate in early trials was relatively low (~26%) (Porter et al, 2015) compared to acute lymphoblastic leukaemia (ALL, 90%) (Maude et al, 2014). A recent study of 41 CLL patients who had undergone CAR-T cell therapy, showed that complete response in 9 patients was associated with higher frequencies of CD8^+CD45RO^−CD27^+ T cells pre-infusion (Fraietta et al, 2018). These were classified as “early effector memory” T cells and suggest that the phenotype of pre-adoptive therapy T cells may determine their capacity to expand and persist for optimal therapeutic effect. Thus, knowledge of the “base-line” T cell status of patients may allow selection of patients most likely to benefit from therapies involving adoptive transfer of T cells.

The current study identified two T cell subsets, CD8^+ and CD4^+PD-1^−HLA-DR^+, which could be used to identify CLL patients at risk of disease progression. These findings will need to be validated on larger cohorts of treatment-naive patients, as the cohort studied included some patients with stable disease who had been previously treated. The results raise the possibility that phenotypic monitoring of T cell subsets could identify patients who might benefit from early intervention and could also be used alongside other clinical parameters for post-therapy monitoring. This might be particularly relevant to therapies involving BTK/PI3K inhibitors or venetoclax for which the long-term effects on T cell immunity are unknown (Man & Henley, 2019).

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Authorship
LE, EW, PH, and SH performed the research and analysed data. RH performed statistical analysis of the data. SM, CP and CF designed and supervised the study. SM wrote the paper, with substantial input from LE, CP and CF. All authors have approved the final version of the manuscript.

Conflict of Interest Disclosure
The authors have nothing to disclose.
References


are driven into a senescent KLRG1+ effector memory phenotype. Cancer Immunology, Immuonotherapy, 62, 1697–1709.


Figure Legends

**Figure 1.** Influence of CD4:CD8 ratio on overall survival and progression-free survival. Kaplan-Meier analysis was used to test two cohorts of CLL patients A) % overall survival (OS) for the patient cohort (n=63) from Nunes et al 2011 was measured in relation to CD4:CD8 ratio. B) % progression-free survival (%PFS) for the validation cohort (n=74) measured in relation to CD4:CD8 ratio. Hazard ratios (HR) with 95% confidence limits and probability are shown for each comparison. Numbers of patients still at risk (No.s at risk) are shown for each time point.

**Figure 2.** Phenotypic comparison of T cells in CLL (n=74) and Healthy Donors (HD, n=14). Freshly isolated PBMC were stained with 2 separate panels of antibodies before analysis with FlowJo (boolean gating) and SPICE software. Panel 1 contained CD57, HLA-DR, PD-1 specific antibodies, while Panel 2 contained CD57, CD38, CD127 and KLRG-1 specific antibodies. A) CD8 T cells. B) CD4 T cells. Each coloured pie segment depicts the number of co-expressed markers (from 0-4), while each coloured arc depicts the expression of individual markers.

**Figure 3.** HLA-DR and PD-1 co-expression on CD4+ T-cells of CLL patients and healthy controls. Freshly isolated PBMC were analysed using flow cytometry and FlowJo software as described in Materials and Methods. A) The %CD4+HLA-DR+PD-1+ T-cells in the whole CD4+ T-cell compartment, CD4+ Effector Memory (EM) and CD4+ Terminally differentiated Effector Memory (EMRA) memory subsets of CLL patients (n = 74) and healthy donors (HD, n = 14). B) Representative flow plots of a CLL patient with a high frequency (38.3%) of CD4+HLA-DR+PD-1+ T-cells and a HD with a low frequency (3.3%). The plots show sequential gating for CD3+ T cells, then CD4+ T cells, followed by selection of HLA-DR+PD-1+ T cells based on thresholds set by FMO controls. Statistical analysis (HD vs. CLL) was performed using the Mann-Whitney test. Levels of significance were indicated as follows; * = p ≤0.05; ** = p ≤0.01; *** = p ≤0.001.

**Figure 4.** Prognosis of CLL patients stratified according to CD8+ T cell frequencies. The %CD8+ T cells were determined by flow cytometry. Multivariate recursive partition analysis was carried out to establish significant cut-off values for %CD8+ T cells within the study cohort. Kaplan-Meier
curves were generated for progression-free survival (%PFS) of CLL patients split into two populations based on (A) 33.2% (median value of cohort) (B) 56.9% (optimal cut-off value identified using recursive partitioning). Hazard ratios (HR) with 95% confidence limits and probability are shown for each comparison. Numbers of patients still at risk (No.s at risk) are shown for each time point.

**Figure 5.** Prognosis of CLL patients stratified by the frequency of CD4⁺PD-1⁺HLA-DR⁺ T cells. Multivariate recursive partition analysis was carried out to establish significant cut-off values for % CD4⁺PD-1⁺HLA-DR⁺ T cells (%CD4PH) within the study cohort. Kaplan-Meier curves for progression free survival (%PFS) were generated after dividing CLL patients into groups according to the following cut-off points: (A) 9.1% (median value of cohort) (B) 14.4% (C) 31.5% (D) 4.95%. Hazard ratios (HR), with 95% confidence limits and probability shown for each comparison. Numbers of patients still at risk (No.s at risk) are shown for each time point.

**Figure 6.** Characterisation of CD4⁺PD-1⁺HLA-DR⁺ T cells from CLL patients.
A. Testing for association between the frequency of CD4⁺PD-1⁺HLA-DR⁺ T cells and CMV serostatus. A subset of the CLL cohort (n=37) was tested for presence of CMV specific IgG antibodies, then divided into 2 groups depending on positive (n=28) or negative serostatus (n=9). The % CD4⁺PD-1⁺HLA-DR⁺ T cells was determined independently by flow cytometry. Bar graphs depict the mean values with minimum and maximum values. B. Relative frequencies of CD4⁺PD-1⁺HLA-DR⁺ T cells and Tregs in CLL patients and controls (CLL patients n=16, HD n=8). The frequencies of both T cell subsets were measured by flow cytometry; Tregs were defined on basis of CD4, CD25hi and FoxP3 co-expression. The differences between CLL patients and HD for each cell type were analysed using the Mann-Whitney test.
Figure 1. Influence of CD4:CD8 ratio on overall survival and progression-free survival. Kaplan-Meier analysis was used to test two cohorts of CLL patients A) % overall survival for the patient cohort (n=63) from Nunes et al 2011 was measured in relation to CD4:CD8 ratio. B) % progression-free survival for the validation cohort (n=74) measured in relation to CD4:CD8 ratio.
**Figure 2.** Phenotypic comparison of T cells in CLL (n=74) and Healthy Donors (n=14). PBMC were stained with 2 separate panels of antibodies before analysis with FlowJo (boolean gating) and SPICE software. Panel 1 contained CD57, HLA-DR, PD-1 specific antibodies, while Panel 2 contained CD57, CD38, CD127 and KLRG-1 specific antibodies. A) CD8 T cells. B) CD4 T cells.
Figure 4. Prognosis of CLL patients stratified according to CD8+ T cell frequencies. The %
CD8+ T cells were determined by flow cytometric analysis. Multivariate recursive partition
analysis was carried out to establish significant cut-off values for %CD8+ T cells within the study
cohort. Kaplan-meier curves for progression free survival of CLL patients were generated
according to (A) 33.2% (median value) (B) 56.9% (value identified as providing highest hazard
ratio from recursive partitioning).
Figure 5. Prognosis of CLL patients stratified by the frequency of CD4^+PD-1^+HLA-DR^+ T cells.

Multivariate recursive partition analysis was carried out to establish significant cut-off values for % CD4^+PD-1^+HLA-DR^+ T cells within the study cohort. Kaplan-meier curves for progression free survival of CLL patients were generated according to (A) 9.1% (median value) (B) 14.4% (value generated by recursive partition analysis) (C) 31.5% (maximum % generated by recursive partition analysis) (D) 4.95% (minimum % generated by recursive partition analysis).
Figure 6. Characterisation of CD4\(^+\)PD-1\(^+\)HLA-DR\(^+\) T cells.
A. Testing association between % CD4\(^+\)PD-1\(^+\)HLA-DR\(^+\) T cells and CMV serostatus in validation cohort (CMV seropositive n=28, CMV seronegative n=9). B. Relative frequencies of CD4\(^+\)PD-1\(^+\)HLA-DR\(^+\) T cells and Tregs in CLL patients and controls (CLL patients n=16, HD n=8). Treg were defined on basis of CD4, CD25\(^{hi}\) and FoxP3 co-expression.