Expansion of a CD8*PD-1* replicative senescence phenotype in early stage CLL patients is associated with inverted CD4:CD8 ratios and disease progression

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Abstract

Purpose: Patients with chronic lymphocytic leukemia (CLL) display immune deficiency that is most obvious in advanced stage disease. Here we investigated whether this immune dysfunction plays a pathological role in the progression of early stage disease patients.

Experimental design: We performed eight-color immunophenotyping analysis in a cohort of 110 untreated early stage CLL patients and 22 age-matched healthy donors and correlated our findings with clinical outcome data.

Results: We found a significant reduction in naïve CD4+ and CD8+ T cells in CLL patients. Only the CD4+ subset showed significantly increased effector memory cells (TEM and TEMRA) in the whole cohort (P=0.004 and P=0.04 respectively). However, patients with inverted CD4:CD8 ratios (52/110) showed preferential expansion of the CD8 compartment with a skewing of CD8+ TEMRA (P=0.03) coupled with increased percentage of CD57+CD28-CD27- T cells (P=0.008), and PD-1 positivity (P=0.027) consistent with a replicative senescence phenotype. Furthermore, inverted CD4:CD8 ratios were associated with shorter lymphocyte doubling time (P=0.03), shorter time to first treatment (P=0.03) and reduced progression-free survival (P=0.005).

Conclusions: Our data show that the emergence of CD8+PD-1+ replicative senescence phenotype in early stage CLL patients is associated with more aggressive clinical disease. Importantly, these findings were independent of tumor cell prognostic markers and could not be accounted for by patient age, changes in regulatory T cell frequency or cytomegalovirus serostatus.

(n = 217)
Translational Relevance

This paper represents the most in-depth, large-scale analysis of T cell subsets in early stage CLL patients yet to be performed. We show, for the first time, that inverted CD4:CD8 ratios are associated with shorter lymphocyte doubling time, shorter time to first treatment and reduced progression-free survival in this disease suggesting that T cell dysfunction contributes towards disease progression. We went on to show that patients with inverted CD4:CD8 ratios demonstrated a preferential expansion of CD8+ terminal effector memory cells coupled with a replicative senescence phenotype. Taken together, our data show that the emergence of a CD8+PD-1+ 'exhaustion' phenotype in early stage CLL patients is associated with a loss of immunological control and more aggressive clinical disease.

(n = 113)
Introduction

Chronic lymphocytic leukemia (CLL) is characterized by immunodeficiency of which hypogammaglobulinemia is the most clinically obvious.(1) However, profound defects in cell-mediated immunity are also a feature of this disease and patients usually have abnormalities in T cell numbers and function.(2-5) Treatment with chemotherapy appears to exacerbate this problem by inducing T lymphopenia thereby rendering patients more susceptible to infection.(6) A number of recent studies have shown abnormalities in the phenotype of CD4 and CD8 T cells, including inversion of the normal CD4:CD8 ratio(2-5) and the accumulation of terminally differentiated effector memory T cells with relative absence of naive precursors.(7,8) By definition, these memory T cells are derived from antigen exposure and it would appear that repeated or chronic antigen stimulation is a feature of this disease. In keeping with this notion, markers of chronic activation are increased on both the CD8+ and CD4+ T cell subsets in CLL patients compared to normal controls.(9) Precisely what antigen(s) are involved in this process remains unresolved, but a number of groups have suggested that cytomegalovirus (CMV) may play a role in driving CD4+ and CD8+ effector memory T cells.(5,10,11) However, CMV accounts for a relatively small number of the total T cell count and CMV seronegative individuals still manifest T cell expansion.(5)

Although the exact mechanism by which T cells accumulate in CLL is not well defined, T cells derived from patients with advanced disease have shorter telomeres suggesting that these T cells are in some way reacting to the growth of the CLL clone.(12) Consistent with this idea, a number of groups have identified a population of anti-
leukemic T cells, these have been the subject of interest especially in the context of immunotherapy.(13-15)

Here we describe the most detailed, large scale, immunophenotypic analysis ever performed on treatment naïve, early stage CLL patients. We demonstrated a skewed distribution of T cell subsets and a preferential expansion of terminally differentiated CD8+ T cells in CLL. Strikingly when patients were stratified into those with inverted (CLLIR) and normal (CLLNR) CD4:CD8 ratios, we observed that the CLLIR group had a preferential expansion of CD8+PD-1+ T cells expressing other markers consistent with replicative senescence. These same patients demonstrated significantly poorer prognosis suggesting that a loss of immunological control has the capacity to alter the pathology of CLL.
Materials and Methods

**Blood samples from healthy volunteers and CLL patients**

110 CLL patients were recruited from clinics at the University Hospital of Wales and Llandough Hospital (age range 48-93 years). Blood samples were collected with informed consent in accordance with the ethical approval obtained from South East Wales Research Ethics Committee (02/4806). A control group of 22 age-matched healthy donors was recruited from local volunteers (age range 42 to 77 years). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Histopaque-1077, Sigma) density gradient centrifugation. 95/110 (86.4%) of the CLL patients were diagnosed in Binet stage A and none had received treatment prior to sample analysis. The median follow-up for the cohort was 4.6 years and during this time 23/110 (21%) required treatment.

**Antibodies**

The following pre-titrated antibodies were used in this study: anti-CD56-FITC, CD16-APC, CD57-FITC (Serotec), CD5-PE-Cy5.5, CD45RO-APC (Invitrogen), CD19-PE-Cy7, CD27-PE-Cy7, FoxP3-FITC (eBioscience), CD8-APC-Cy7, CD4-Pacific blue, CD3-AmCyan, TCRγδ-APC, CD28-percp-cy5.5 (BD Biosciences), CCR7-PE (RnD Systems), CD25-PE (Miltenyi Biotec) and PD-1-FITC (BD Biosciences).

**Immunofluorescent staining and flow cytometric analysis**

Direct immunofluorescent staining was performed by adding conjugated antibodies to cells for 15 minutes at 4°C in the dark, before being washed twice with 1% FCS-PBS. Between 5x10^5 to 1.5x10^6 cells were used per tube and were analysed immediately.
after staining without fixation. For the Treg panel, cells were resuspended in 100μl of 1% FCS-PBS and stained for 30 minutes on ice with the surface cell markers: CD25-PE, CD45RA-APC, CD4-Pacific blue, and CD3-AmCyan. Cells were washed twice with 500μl cold 1% FCS-PBS. FOXP3 staining was performed using the FITC anti-human FOXP3 staining kit (eBioscience). Compensation was performed automatically using an anti-Mouse Ig/Negative control compensation particles set (BD) and FACSDiva software. Fluorochrome-conjugated monoclonal antibodies (with the same concentration added to the cell samples) were used as single-colour compensation controls. Cells were analysed using a BD FACSCanto II cytometer (BD) and analysis was performed using FlowJo analysis software. Analysis of PBMC by flow cytometry involved the construction of a live lymphocyte gate that was based upon the forward and side scatter profiles of these cells. CD4:CD8 ratios were considered inverted if the ratio was ≤1.0. This threshold was selected based on the median CD4:CD8 ratio of the cohort but also represents the point at which the numbers of CD8+ cells outnumber CD4+ cells.

**CMV serostatus**

CMV (IgG) serostatus was determined in a blinded fashion by the local Public Health Laboratory Service in a subset of 40 of the patient cohort, 20 inverted ratio samples (CLLIR) and 20 normal ratio samples (CLLNR).

**Statistics**

Non-parametric Mann-Whitney U test was used for comparison of two independent groups. Analysis of prognostic subsets was performed using Fisher’s exact test or Chi-square. Time to first treatment and progression-free survival time were calculated from
date of diagnosis and curves were constructed using the method of Kaplan and Meier. Statistical analysis was carried out using Prism 5.0 (GraphPad Software, San Diego, CA).
Results

Evaluation of peripheral blood lymphocyte populations

As a first step, we compared the frequency of peripheral blood lymphocyte populations in CLL patient samples and age-matched healthy donors (Supplementary Table 1). The percentages of B cells (CD19+), malignant CLL B cells (CD19+ CD5+), T cells (CD3+ CD4+ and CD8+), innate NK cells (CD56+ CD16+), γδ T cells (CD3+ TCRγδ+) and Tregs (CD4+ CD25high Foxp3+) were analyzed. We found increased percentages of γδ T cells (P=0.05) and Tregs (P=0.03) in CLL patients compared to healthy donors along with significantly decreased percentages of CD3+ T cells (P<0.0001) and NK cells (P<0.0001). An increase in the percentage of CD8+ T cells among CD3+ T cells was also observed in CLL patients (P=0.004), while the percentage of CD4+ T cells among the CD3+ T cells was significantly reduced (P<0.0001). On closer evaluation, there was a marked differential expansion of CD8+ T cells in 52 patients that resulted in the inversion of their CD4:CD8 ratio (CLLIR). Absolute counts were available for 25/52 of the CLLIR patients. Comparison with 25 normal CD4:CD8 ratio (CLLNR) patients and 10 age-matched healthy controls revealed that, consistent with previous findings,(5) the numbers of both CD4+ and CD8+ T cells were increased in CLL patients compared to normal age-matched controls. Furthermore, the inverted CD4:CD8 ratio patients showed no evidence of a decline in CD4+ T cell numbers, rather the inversion of the CD4:CD8 ratio was caused by the preferential expansion of CD8+ T cells (Supplementary Table 2).
Comprehensive analysis of T cell subsets in CLL

It is now accepted that a skewed maturation of T cells occurs with ageing and disease, including chronic viral infection.\(^\text{16-18}\) In the first instance, we evaluated whether the frequency of CD4\(^+\) and CD8\(^+\) memory T cell subsets differed between CLL patients and age-matched controls. The T cell subsets were defined by dividing both the CD4 and CD8 compartments on the basis of CD45RO and CCR7 expression into naïve (CCR7\(^+\)CD45RO\(^-\)), central memory (T\(_{\text{CM}}\); CCR7\(^+\)CD45RO\(^+\)), effector memory (T\(_{\text{EM}}\); CCR7\(^-\)CD45RO\(^-\)) and terminally differentiated effector cells (T\(_{\text{EMRA}}\); CCR7\(^-\)CD45RO\(^-\)). Supplementary Figure 1 shows the gating strategy employed in this study to define T cell subsets. Figure 1A shows that there was a significant reduction in the percentage of naïve CD8\(^+\) subsets in CLL patient samples (P=0.0001) with a trend towards an increased frequency of T\(_{\text{EM}}\) and T\(_{\text{EMRA}}\) in the CD8\(^+\) compartment. Figure 1B shows a similar pattern in the CD4\(^+\) compartment; reduced frequency of naïve (P=0.03) and central memory (P=0.04) subsets and an increase in T\(_{\text{EM}}\) (P=0.004) and T\(_{\text{EMRA}}\) subsets (P=0.04). On the basis of these results, the greatest differences in T cell subsets between CLL patients and healthy age-matched controls appear to be found in the CD4\(^+\) compartment.

CD8\(^+\) cells from patients with Inverted CD4:CD8 ratios have an increased terminal differentiation effector phenotype

A more detailed analysis of our cohort revealed that 47% (52/110) showed a marked preferential expansion of CD8\(^+\) cells which resulted in an inversion of the normal CD4:CD8 ratio. We therefore divided our cohort into CLL\(_{\text{NR}}\) and CLL\(_{\text{IR}}\) subsets based on a CD4:CD8 ratio threshold of 1.0. This threshold was derived from the median CD4:CD8 ratio for the entire cohort (Figure 2A) but a ratio of ≤1.0 also represents the point at
which the CD8 compartment becomes larger than the CD4 compartment. We subsequently reanalyzed the T cell subsets in these groups. Figure 2B shows significant reductions in the naïve and central memory CD8$^+$ subsets in CLL$^{IR}$ patients ($P=0.002$ and $P=0.004$ respectively) when compared with CLL$^{NR}$ patients. Furthermore, there was a significant increase in the T$_{EMRA}$ subset in the CLL$^{IR}$ group ($P=0.03$). Figure 2C shows that naïve CD4 cells were also significantly reduced in the CLL$^{IR}$ subset ($P=0.0007$) with a concomitant increase in T$_{EM}$ ($P=0.0005$) but not T$_{EMRA}$ ($P=0.32$) in this compartment. Figures 2D and 2E show the skewing of the CD8 and CD4 subsets in CLL$^{IR}$ and CLL$^{NR}$ samples compared to normal age-matched controls. Most notably, we observed a preferential relative expansion in CD8$^+$ EMRA ($P = 0.03$) and a concomitant decrease in CD8$^+$ naïve and central memory subsets ($P = 0.002$ and $P = 0.004$ respectively) in the CLL$^{IR}$ subset. Consistent with previous findings,$^{(19)}$ the inversion of the CD4:CD8 ratio was not associated with the age of the patients since the median age of the CLL$^{NR}$ and CLL$^{IR}$ subsets were not significantly different ($P=0.47$).

**CLL$^{IR}$ is associated with inferior prognosis in CLL**

Intuitively, the increased percentage of effector T cell subsets seen in CLL, particularly in the CLL$^{IR}$ group, might be beneficial either because of increased ability to provide protection against pathogens or perhaps increased immunological control over the tumor cells. Therefore, we evaluated whether an inverted CD4:CD8 ratio had any prognostic relevance in CLL. Contrary to our expectations, the CLL$^{IR}$ group had significantly shorter time to first treatment (Figure 3A; $P=0.03$) and progression-free survival (Figure 3B; $P=0.005$) when compared to the CLL$^{NR}$ group. Therefore, an inverted CD4:CD8 ratio appears to confer an inferior clinical prognosis in this early stage cohort of CLL patients.
**CLL**<sup>IR</sup> patients show increased frequency of a CD57<sup>+</sup>CD28<sup>-</sup>CD27<sup>-</sup> phenotype

In an attempt to rationalize the poor prognosis associated with an inverted CD4:CD8 ratio, we performed a more detailed analysis of the T cell subsets in our cohort. The emergence of a CD57<sup>+</sup>CD28<sup>-</sup>CD27<sup>-</sup> phenotype is associated with replicative senescence and has been observed with ageing and in diseases including CLL. We therefore analyzed CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets for the co-expression of CD57, and the absence of CD27 and CD28 to elucidate whether this phenotype was more pronounced in CLL<sup>IR</sup> and CLL<sup>NR</sup> patients compared to age-matched healthy donors. Figure 4A shows that a CD57<sup>+</sup>CD28<sup>-</sup>CD27<sup>-</sup> phenotype was significantly enhanced in CD8<sup>+</sup> T<sub>EM</sub> cells of CLL patients compared with healthy donors (P=0.019). Furthermore, when we split the cohort into CLL<sup>NR</sup> and CLL<sup>IR</sup> subsets (Figure 4B) we demonstrated a significant increase in CD57<sup>+</sup>CD28<sup>-</sup>CD27<sup>-</sup> expressing cells in both the T<sub>EM</sub> (P=0.0007) and T<sub>EMRA</sub> subsets (P=0.008) in the CLL<sup>IR</sup> group. The CD4<sup>+</sup> T cells also showed an increase in CD57<sup>+</sup>CD28<sup>-</sup>CD27<sup>-</sup> phenotype in the T<sub>EM</sub> subset (Figure 4C; P=0.004) and again this was most noticeable in the CLL<sup>IR</sup> group (Figure 4D; P=0.02). Differences in CD4<sup>+</sup> T<sub>EMRA</sub> were not evaluated due to the very low percentage of this subset in CD4<sup>+</sup> T cells. In accordance with previous findings, this suggests that there is an accumulation of terminally differentiated T cells, particularly in the CLL<sup>IR</sup> group, supporting the concept of chronic activation of the T cell compartment. A previous report suggested that the accumulation of CD8<sup>+</sup>CD57<sup>+</sup>CD27<sup>-</sup> T cells in CLL was caused by the expansion of CMV-specific T cells in CMV seropositive patients. We therefore serotyped 20 CLL<sup>IR</sup> and 20 CLL<sup>NR</sup> patients to assess whether this phenotype was linked to CMV serostatus. 17/20 of the CLL<sup>IR</sup> group were CMV seropositive. However, 15/20 of the CLL<sup>NR</sup> group were also CMV seropositive. This indicates that CMV is not solely responsible for the preferential expansion of CD8<sup>+</sup>CD57<sup>+</sup>CD28<sup>-</sup>CD27<sup>-</sup> T cells in the CLL<sup>IR</sup> group. In keeping
with this notion, there was no significant percentage increase in either CD8+ cells (P=0.75) or CD8+CD57+CD28-CD27- T cells (P=0.43) in the CMV seropositive subset when compared with the seronegative subset (Figure 4E).

**CD8+ PD-1 expression is associated with inverted CD4/CD8 ratios**

Given that the differential expansion of CD8+ T cells in the inverted CD4/CD8 ratio group was characterized by a replicative senescence phenotype, we went on to analyze the expression of PD-1 in 77/110 (70%) of our patient cohort. PD-1 expression has recently been associated with a CD8+ T cell exhaustion phenotype in mice with disseminated acute myelogenous leukemia,(24) we therefore investigated whether this marker was differentially expressed in the CLLIR group. Figure 5A shows that there were significantly more PD-1+CD8+ T cells in the CLLIR group when compared with the CLLNR group (P=0.027). Furthermore, the percentage of PD-1+CD8+ T cells was not associated with CMV serostatus (Figure 5B; P=0.63). It is important to note that PD-1 expression was significantly lower in CD4 and CD8 T cell subsets in normal age-matched controls when compared to CLL samples (Supplementary Figure 3).

**CLLIR is not associated with preferential reduction in Tregs**

In this study we found a significant increase in Tregs in CLL patients when compared with healthy donors (P=0.03). This is in accordance with previous reports that show that the frequency of Tregs is higher in CLL patients and is further increased in patients with advanced stage disease.(25,26) We therefore analyzed whether there was a significant difference in the frequency of Tregs in CLLIR patients when compared to CLLNR patients (Figure 6A). We found no significant difference in the percentage of
Tregs in the inverted ratio group (P=0.46) suggesting that changes in Tregs cannot explain the prognostic significance of inverted CD4:CD8 ratios.

**CLLIR is not associated with tumor cell phenotype or Binet stage at diagnosis**

In order to establish whether there were confounding factors that could explain the prognostic significance of inverted CD4:CD8 ratios in our CLL cohort, we assessed whether there was an association between this phenomenon and known tumor cell prognostic markers (Figures 6B-F). We found no significant difference in CD38 expression (P=0.8), ZAP-70 expression (P=0.15) *IGHV* mutation status (P=0.55), or Binet stage at diagnosis (P=0.51) between the CLL^NR^ and CLL^IR^ groups. However, we did show an association between inverted CD4:CD8 ratio and lymphocyte doubling time less than 12 months (P=0.03) suggesting a role for T cells in the immunological control of tumor cell expansion in this disease.
Discussion

In this study, we performed a comprehensive analysis of T cell subsets in a large cohort of 110 untreated CLL patients. Most of our analysis was based on percentage expression of specific phenotypic subsets but this comparative analysis was also supported by absolute counting data in a subset of the cohort. In keeping with previous studies,(27-31) we found that the T cell subset composition was skewed towards a memory phenotype with a reduced CD4:CD8 ratio in 52% of the patients. Inverted CD4:CD8 ratios have been previously documented in CLL,(2) but here we show for the first time that inverted CD4:CD8 ratios are associated with a shorter lymphocyte doubling time and confer an inferior prognosis in early stage disease patients. Importantly, this was independent of patient age, tumor-associated prognostic markers, percentage of Tregs and CMV serostatus. A recent paper by Gonzalez-Rodriguez et al.(4) appears to contradict our findings in terms of the prognostic impact of CD8 T cells. However, this study did not assess the clinical impact of comparative changes in CD4 and CD8 subsets. Instead, they evaluated the CD8:tumor B cell ratio and concluded that higher ratios conferred a good prognosis. This is not surprising given that this ratio is influenced predominantly by the tumor burden, so their findings are not directly comparable to the ones reported in our study.

As reported previously,(32) CD4+ T cell subsets were significantly skewed in our CLL cohort when compared to normal age-matched controls and this was most apparent in the CLLIR subset. However, absolute T cell count analysis of the CLLIR group revealed that the inversion was caused by the preferential expansion of the CD8+ compartment rather than a relative reduction in CD4+ cells (Supplementary Table 2). Further
examination of CD8+ T cell subsets revealed a significant skewing towards T_{EMRA} cells in the CLL_{IR} subset; a phenomenon that has been previously reported in a very small cohort of 11 CLL patients.(30) Our study confirms and extends those observations in a cohort of 110 patients and demonstrates that expansion in CD8+ T_{EMRA} is only observed in the CLL_{IR} patients.

Down regulation of CD28 and increased expression of CD57 is associated with replicative senescence that can lead to the inability of T cells to proliferate and an enhanced tendency to apoptosis.(9,20,21,33,34) The development of this phenotype can occur in response to repeated/chronic antigenic stimulation and has been associated with ageing. Several lines of evidence suggest that the alteration in the T cell subsets in both CLL patients and healthy older individuals can be associated with chronic antigenic stimulation by viral infections, such as CMV.(10,11,35) Therefore, the expansion of T_{EM} and T_{EMRA} might be related to the fact that sub-clinical CMV infection is common in CLL and this can drive chronic stimulation and exhaustion in the T cell compartment.(10) However, here we present evidence that the preferential expansion of the CD8+ compartment in CLL_{IR} patients cannot be solely explained by CMV infection. Our data confirm that CMV seropositivity is a common phenomenon in CLL but it was almost as prevalent in the CLL_{NR} group and so the preferential expansion of CD8+ T cells and the increase in CD58+CD28-CD27- T_{EMRA} in the CLL_{IR} subset cannot be attributed to CMV alone. These findings are in keeping with a recent report that showed CD8+ expansion in CMV seronegative individuals.(5) Further evidence for a CMV-independent process driving the emergence of replicative senescence phenotype was derived from our assessment of PD-1 expression in CD8+ T cells. PD-1 was significantly increased in CLL_{IR} patients but was not associated with CMV serostatus.
Intuitively, large numbers of effector T cell subsets should be beneficial because of their ability to provide immediate immunity. Thus our findings associating increased CD8+ T cell effector numbers with inferior clinical prognosis may appear somewhat paradoxical. However, the depletion of naïve and central memory CD8+ T cells and the expansion of T_{EM} and T_{EMRA} cells may hamper long-term immunoprotection as there is a reduction in the diversity and functional integrity of the T cell subsets. The increase of CD57+CD28-CD27- effector T cells, coupled with increased PD-1 expression, indicates that a large proportion of the effector cells are highly differentiated and in a state of replicative senescence and thus have the least proliferative potential amongst the T cell subsets. This contributes to a decrease competence to effectively respond to re-infection or maintain memory for tumor antigens expressed by relapsing tumor cells. Moreover, a decrease in the relative proportion of naïve T cells will inevitably decrease the diversity of the naïve T cell receptor repertoire. This can affect the capacity of CLL patients to resist \textit{de novo} infections or to respond to the appearance of new tumor antigens.

Tumor antigens might drive the preferential expansion of CD8+ T cells in the CLL\textsuperscript{R} patients. Proliferation of T cells against autologous CLL tumor cells has been demonstrated \textit{in vitro},(12,14,36,37) and this is likely to reflect a composite response against multiple tumor antigens rather than a response restricted to a single immunodominant antigen. This concept of T cell expansion driven by CLL tumor cells, appears to be at odds with the suppressive properties of CLL cells. Laboratory studies have demonstrated a defective immune synapse between T cells and CLL cells(38) and the ability of CLL cells to manipulate the gene expression profile of T cells through cell-
cell contact. However, tolerance of CD8+ T cells can be accompanied by proliferation, and expanded populations of anergic or non-functional CD8+ T cells can be found in human cancer and chronic infection. This suggests the possibility that CLL tumor cells can induce the proliferation of CD8+ T cells that become incapable of initiating, continuing or completing an immune response against the leukemic B cells, and other antigens, and thus may be involved directly in sustaining the tumor. However, the ability to mount a response against CMV appears to be maintained in CLL patients as CMV reactivation is not common except for those treated with alemtuzumab.

Taken together, our results suggest that the inversion of the CD4:CD8 ratio in treatment naïve, CLL patients is associated with the preferential expansion of CD8+PD-1+ highly differentiated memory cells with a replicative senescence phenotype. Crucially, the emergence of these cells appear to signify a loss of immunological control against the leukemia since patients with CLLIR have shorter lymphocyte doubling times, more progressive disease and require earlier treatment.

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Author contributions

C.N. Performed research, analysed data
R.W. Performed research, analysed data
M.M. Contributed vital new reagents and revised the paper

C.F. Designed research, contributed vital reagents and revised the paper

S.M. Designed research, analyzed data and wrote the paper

C.P. Designed research, analyzed data and wrote the paper

**Conflict of interest disclosure**

The authors have no conflicts of interest.
References


increased abnormal expression by advancing stage. Leukemia. 2003; 17(11): 2252-2254.


Figure Legends

**Figure 1.** **CD8**⁺ and **CD4**⁺ T cell subsets of CLL patients and healthy volunteers. The number of positive cells was determined by flow cytometry (FACS CantoII). Lymphocytes were gated based on their forward and side scatter profile. T cells were gated on CD3⁺CD8⁺ or CD3⁺CD4⁺ cells. T cell subsets were defined based on the expression of CCR7 and CD45RO: Naive (CCR7⁺CD45RO⁻), Central Memory (T_{CM}) (CCR7⁺CD45RO⁺), Effector Memory (T_{EM}) (CCR7⁻CD45RO⁻), and Effector (T_{EMRA}) (CCR7⁻CD45RO⁻). (A) Percentage of each subset in total CD8⁺ T cells of CLL patients and healthy donors. (B) Percentage of each subset in total CD4⁺ T cells of CLL patients and healthy donors. The mean within each group is shown. Flow cytometric analysis and statistical analysis (Mann Whitney test) were carried out using FlowJo software and GraphPad Prism, respectively. CLL patients (filled symbols) and age-matched healthy donors =HD (open symbols).

**Figure 2.** **CD8**⁺ and **CD4**⁺ T cell subsets in **CLL**{IR}, **CLL**{NR} patients and healthy volunteers. (A) CD4:CD8 ratios were determined for all 110 CLL patients. The median ratio was 1.0 for the whole cohort. (B) Percentage of each subset in total CD8⁺ T cells of **CLL**{IR}, **CLL**{NR} patients and healthy donors. (C) Percentage of each subset in total CD4⁺ T cells of **CLL**{IR}, **CLL**{NR} patients and healthy donors. The values in each pie charts, (D) and (E), represent the mean frequency of each subset in the CD8⁺ or CD4⁺ T cell population for **CLL**{IR}, **CLL**{NR} patients and age-matched healthy donors respectively.

**Figure 3.** Kaplan Meier curves for time to first treatment and progression-free survival for **CLL**{IR} and **CLL**{NR} patients. Kaplan-Meier analysis was used to determine the influence of CD4:CD8 ratio on (A) time to first treatment and (B) progression-free survival in CLL patients. A log-rank test was used to test the difference between the two curves.

**Figure 4.** **CD57**⁺ **CD27**⁻**CD28**⁻ EM and EMRA expression in total CD8⁺ T cells and CD4⁺ T cells of CLL patients and healthy volunteers. (A) Percentage of CD57⁺CD27⁻CD28⁻ T_{EM} and T_{EMRA} in total CD8⁺ T cells of whole CLL group of patients and age-matched healthy volunteers. (B) Percentage of CD57⁺CD27⁻CD28⁻ T_{EM} and
T_{EMRA} in CD8^+ T cells of CLL{superscript}IR, CLL{superscript}NR patients and healthy donors. (C) Percentage of CD57^+CD27^-CD28^- T_{EM} in total CD4^+ T cells CLL patient cohort and healthy volunteers. (D) Percentage of CD57^+CD27^-CD28^- T_{EM} in CD4^+ T cells in the CLL{superscript}IR, CLL{superscript}NR patients and healthy donors. The T_{EMRA} subset was not included for CD4^+ T cells due to low percentage of this subset in total CD4^+ T cells. (E) Shows the CMV serostatus has no effect on the percentage of CD8 cells or the percentage of CD57^+CD27^-CD28^- in our CLL cohort.

**Figure 5.** PD-1^+CD8^+ T cells in CLL{superscript}IR and CLL{superscript}NR patients and CMV seropositive and CMV seronegative patients. PD-1 expression was assessed in CD8^+ T cells and was expressed as percentage PD-1 positive cells above isotype control (A) Shows that PD-1 is expressed on significantly more CD8^+ T cells in samples derived from CLL{superscript}IR patients. (B) In contrast, there was no significant increase in the percentage of CD8^+ T cells expressing PD-1 in CMV seropositive samples.

**Figure 6.** Correlation of CD4:CD8 ratio with Tregs, CD38 expression, ZAP-70 expression, IGHV gene mutation status, Binet stage at diagnosis and lymphocyte doubling time. (A) Comparison of the percentage of Tregs in the CLL{superscript}IR, CLL{superscript}NR and age-matched healthy donors (B) Percentage of CD38 expression in CLL{superscript}IR and CLL{superscript}NR. (C) Percentage of ZAP-70 expression in CLL{superscript}IR and CLL{superscript}NR. (D) Percentage unmutated and mutated IGHV genes in the CLL{superscript}IR and CLL{superscript}NR groups. (E) Number of CLL{superscript}IR and CLL{superscript}NR patients in Stage A, B and C. (F) Lymphocyte doubling time in CLL{superscript}IR and CLL{superscript}NR.
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