Chronic lymphocytic leukemia is characterized by impaired immune functions largely due to profound T-cell defects. T-cell functions also depend on co-signaling receptors, inhibitory or stimulatory, known as immune checkpoints, including cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and programmed death-1 (PD-1). Here we analyzed the T-cell phenotype focusing on immune checkpoints and activation markers in chronic lymphocytic leukemia patients (n=80) with different clinical characteristics and compared them to healthy controls. In general, patients had higher absolute numbers of CD3+ cells and the CD8+ subset was particularly expanded in previously treated patients. Progressive patients had higher numbers of CD4+ and CD8+ cells expressing PD-1 compared to healthy controls, which was more pronounced in previously treated patients ($P=0.0003$ and $P=0.001$, respectively). A significant increase in antigen-experienced T cells was observed in patients within both the CD4+ and CD8+ subsets, with a significantly higher PD-1 expression. Higher numbers of CD4+ and CD8+ cells with intracellular CTLA-4 were observed in patients, as well as high numbers of proliferating (Ki67+) and activated (CD69+) CD4+ and CD8+ cells, more pronounced in patients with active disease. The numbers of Th1, Th2, Th17 and regulatory T cells were substantially increased in patients compared to controls ($P<0.05$), albeit decreasing to low levels in pre-treated patients. In conclusion, chronic lymphocytic leukemia T cells display dysregulated expression of immune checkpoints and activation markers in chronic lymphocytic leukemia patients in different ways.
Immune checkpoints and activation markers in CLL

Isolation of peripheral blood mononuclear cells and cell culture conditions

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by density gradient centrifugation on a Ficoll-Hypaque gradient (GE Healthcare, Uppsala, Sweden) and washed twice with Dulbecco’s Phosphate-Buffered Saline 0.9% (DPBS) (Gibco, Life Technologies, Carlsbad, CA, USA). Cells were freshly used or stored in liquid nitrogen until use. After thawing, PBMC were analyzed immediately unless used for stimulation experiments.

3x10^6 PBMC were cultured for 72 hours in humidified air with 5% CO_{2} at 37°C in RPMI 1640 medium (GIBCO, Life Technologies, Carlsbad, CA, USA) supplemented with heat-inactivated autologous serum for fresh samples and pooled normal human AB^+ serum for frozen samples in the presence of phytohemagglutinin (10 μg/mL) (PHA-M, Sigma Aldrig, St. Louis, MO, USA). PBMC cultured in medium alone were used as controls.

Flow cytometric analysis of PBMC

Peripheral blood mononuclear cells were washed with CSB (BioLegend). The following antibodies were used: CD19-AF488 and -PE-Cy7, CD16/CD56-PE, CD4-PerCP, -FITC and -AF700, CD8-APC and -AF700, CD5-PE and -PerCP, CD45RO-FITC, CD25-APC, CD45RA-AF488, PD-1 (CD279)-PE, CD69-AF488, CCR7 (CD197)-AF647, PD-L1 (CD274)-PE, HLA-DR-PerCP, CCR6 (CD196)-AF700, CCR4 (CD194)-PE, CCR5 (CD195)-PE, CCR7 (CD197)-AF647, PD-L1 (CD274)-PE (BD-Biosciences) and the appropriate isotype controls. Further details are provided in the Online Supplementary Appendix.

Sequence analysis of IGHV–IGHD–IGHJ rearrangements

IGHV-IGHD-IGHJ rearrangements were determined through PCR amplification, Sanger sequencing and subsequent sequence interpretation following established international guidelines and using the IMGT® databases and the IMGT/V-QUEST tool (http://www.imgt.org), as previously reported. IGHV gene mutational status was defined as either mutated or unmutated based on the clinically relevant 98% cut-off value for identity to the closest germline gene. Subset #2 cases (IGHV3-21/IGLV3-21 usage, mixed IGHV mutation status) are listed together with IGHV-unmutated cases since this entity is a recognised adverse-prognostic group.

Statistical analyses

Statistical analyses were performed using the GraphPad Prism software 6.0 (GraphPad Software, La Jolla, CA, USA). All tests were two-sided, and P<0.05 was considered statistically significant.

Results

CLL patients had higher absolute numbers of T cells and the number of CD8^+ T cells was related to treatment

Chronic lymphocytic leukemia patients had higher numbers of CD3^+ cells compared to controls (Online Supplementary Table S1); the difference was statistically significant for all the patient subgroups. No difference was observed for CD4^+ cells, while CD8^+ cells were higher in pre-treated progressive patients compared to controls as well as non-progressive (P=0.02 and P=0.001, respectively), irrespective of type of treatment (alumetuzumab or not; fludarabine/cyclophosphamide or not) and IGHV mutational status (data not shown).
PD-1 expression was increased in T cells from pre-treated progressive CLL patients

Compared to controls, CLL patients had higher numbers of PD-1-expressing CD4+ T cells, which related to disease activity and previous treatment. No difference was observed between non-progressive patients and controls (median 258 vs. 169/μL; P=0.1), while progressive patients had higher numbers compared to controls (median 315 and 521/μL for untreated and treated patients; P=0.01 and P=0.0003, respectively). This was observed regardless of IGHV mutational status. Pre-treated patients with progressive disease had higher numbers of PD-1+CD4+ cells as compared to non-progressive (P=0.008) (Figure 1A). There was a moderate positive correlation between PD-1+ CD4+ T cells and total lymphocyte count (r=0.36, P=0.001) (Figure 1D). No expression of PD-L1 on CLL cells was noted (data not shown).

Progressive CLL patients had an increase in PD-1+ antigen-experienced T cells

A subset of patients (n=33) was analyzed for the distribution of CD4+ and CD8+ memory T cells. By CD45RA and CCR7 staining, T-cell subpopulations were identified as naïve (CD45RA+/CCR7+), central memory (CD45RA−/CCR7+), effector memory (CD45RA−/CCR7−), and effector (CD45RA+/CCR7−). CLL patients had higher absolute numbers of CD4+ effector memory cells compared to controls in both untreated and pre-treated patients, but in this latter group, this held true only for patients who had received alemtuzumab, who had higher numbers of CD8+ effector memory and effector cells in progressive patients compared to controls. This was observed in both untreated and pre-treated patients, but in this latter group, this held true only for patients who had received alemtuzumab, who had higher numbers of CD8+ effector memory and effector cells.
memory (P=0.001) and effector (P=0.007) cells compared to controls. Untreated patients with progressive disease had higher numbers compared to non-progressive. CLL patients and controls had comparable numbers of naïve T cells, which were significantly reduced in pre-treated compared to untreated patients. No difference was noted with regard to the numbers of central memory T cells (Figure 2B).

PD-1 expression within the CD4+ population was higher among memory T-cell subsets in patients as compared to controls, with the exception of previously treated patients in which the numbers of PD-1+CD4+ naïve, central memory and effector cells expressing PD-1 were similar to controls (Figure 2C). CLL patients irrespective of disease phase and previous treatment had higher numbers of CD8+ effector memory and effector cells expressing PD-1 compared to controls. The numbers of PD-1+CD8+ naïve cells were low in CLL patients, but higher in untreated patients compared to controls (P=0.03 and P=0.01 for non-progressive and progressive untreated, respectively) (Figure 2D).

**CTLA-4 was only detected intracellularly in CLL T cells**

No expression of surface CTLA-4 was seen in either CD4+ or CD8+ cells from CLL patients and controls. Intracellular CTLA-4 was, however, expressed in a higher number of CD4+ T cells in CLL patients as compared to controls (median 329/µL for non-progressive patients, 717/µL for progressive untreated and 317/µL for progressive pre-treated vs. 136/µL for controls; P<0.001) (Figure 3A). Numbers of CD8+ cells with intracellular CTLA-4 expression were higher both in patients treated with alemtuzumab (P=0.001) and cyclophosphamide/fludarabine (P=0.0007) compared to controls. A positive correlation was observed between the numbers of CD4+ T cells with intracellular CTLA-4 and total lymphocyte count (r=0.50, P=0.003). CLL patients also had higher numbers of CD8+ cells with intracellular CTLA-4 compared to controls (median 23/µL for non-progressive patients, 79/µL for progressive untreated and 59/µL for progressive pre-treated vs. 7.5/µL for controls; P<0.0001). Both untreated and previously treated patients with progressive disease had higher numbers of CD8+ cells with intracellular CTLA-4 as compared to non-progressive (P<0.05) (Figure 3B), which correlated positively with the total lymphocyte count (r=0.38, P=0.03).

**T cells from progressive CLL patients displayed an activated phenotype but no expression of the co-stimulatory molecule CD137**

Lower numbers of CD69+CD4+ cells were noted in non-progressive compared to progressive CLL patients irrespective of previous treatment (median 30/µL in untreated
and 22/μL in pre-treated compared to 5/μL in non-progressive; \( P=0.002 \) (Figure 4A). Non-progressive patients had lower numbers of CD69^+CD8^+ cells compared to controls (median 6/μL in non-progressive and 27/μL in controls; \( P=0.008 \)), untreated progressive (median 16.5/μL; \( P=0.009 \)) and previously treated progressive (median 40/μL; \( P=0.0003 \)) patients (Figure 4B). A moderate positive correlation was observed between the total lymphocyte count and the numbers of CD69^+CD4^+T cells (\( r=0.39, P=0.0004 \)) and CD69^+CD8^+ T cells (\( r=0.34, P=0.002 \)). No expression of CD137 was observed on T cells from CLL patients and controls (data not shown).

**Expression of immune checkpoints and activation markers could be induced on CLL T cells**

It is known that T-cell stimulation leads to upregulation of immune checkpoints and activation markers on the cell surface.8-10,20,21 We, therefore, stimulated T cells from CLL patients and controls for 72 hours with PHA. This method was chosen rather than others of unspecific T-cell stimulation to more closely reflect physiological conditions and avoid interference with the flow-cytometry staining.

PD-1 expression increased markedly on both CD4^+ and CD8^+ cells, and similarly in CLL patients and controls (Online Supplementary Figure S1A). Surface CTLA-4, which was virtually absent at baseline both in CLL patients and controls, was induced on CD4^+ cells both from CLL patients and controls (median % CTLA-4^+CD4^+ cells after PHA stimulation was 2.9 in non-progressive, 14.9 in progressive patients and 4.8 in controls) (Online Supplementary Figure S1B). CD69 expression also increased in both CD4^+ and CD8^+ cells and to a similar degree in CLL patients and controls (Online Supplementary Figure S1C), while CD137 expression increased to a higher extent in T cells from progressive patients compared to controls (\( P=0.03 \) and 0.01 for the CD4^+ and CD8^+ cells, respectively) (Online Supplementary Figure S1D). We also studied expression of CD103, a marker for alloantigen-induced CD8^+ Tregs20 and found that the percentage of CD103^+CD8^+ T cells increased more in controls than in CLL patients (median increase 4.8% in controls vs. 0.7% in non-progressive and 0% in progressive patients; \( P=0.01 \) and 0.004, respectively) (Online Supplementary Figure S1E).

**Proliferating T cells were significantly higher in CLL patients compared to controls and correlated with disease activity**

The percentage of proliferating (Ki67^+) circulating tumor cells (CD5^+CD19^+) in CLL patients was low (<1%) irrespective of disease activity and previous treatment (data not shown). However, CLL patients had higher absolute numbers of proliferating CD8^+ T cells compared to con-
Table 2. Summary of the different T-cell subpopulations and T cells expressing immune checkpoints or activation / proliferation markers as compared between the different studied subject groups. (A) CD4+ T cells. (B) CD8+ T cells.

### A CD4+ T cells

| Disease | Effect of disease phase | Treatment |
|---------|-------------------------|-----------|
| non-progressive CLL vs. healthy controls | progressive untreated vs. non-progressive | progressive pre-treated vs. progressive untreated |
| CD4+ | ↔ | ↔ | ↔ |
| PDL1+ | ↔ | ↔ | ↔ |
| T hare | ↔ | ↔ | ↓ (***) |
| T EM | ↑ (***) | ↔ | ↓ (*) |
| T EMRA | ↔ | ↔ | ↔ |
| i.c. CTLA-4+ | ↑ (****) | ↔ | ↔ |
| CD68+ | ↔ | ↑ (**) | ↔ |
| Th1 | (****) | ↔ | ↓ (****) |
| Th2 | ↑ (*) | ↔ | ↓ (*) |
| Th17 | ↔ | ↔ | ↓ (**) |
| Tregs | ↑ (*) | ↔ | ↔ |
| Ki67+ | ↔ | ↔ | ↔ |

### B CD8+ T cells

| Disease | Effect of disease phase | Treatment |
|---------|-------------------------|-----------|
| non-progressive CLL vs. healthy controls | progressive untreated vs. non-progressive | progressive pre-treated vs. progressive untreated |
| CD8+ | ↔ | ↔ | ↔ |
| PDL1+ | ↔ | ↔ | ↔ |
| T hare | ↔ | ↔ | ↓ (***) |
| T EM | ↔ | ↔ | ↔ |
| T EMRA | ↔ | ↑ (***) | ↔ |
| i.c. CTLA-4+ | ↑ (****) | ↑ (*) | ↔ |
| CD68+ | ↓ (**) | ↑ (**) | ↔ |
| Ki67+ | ↑ (*) | ↑ (*) | ↔ |

Statistically significant differences are symbolized as follows: ↔: no difference; ↑: higher; ↓: lower. i.c.: intracellular. *P<0.05, **P<0.005, ***P<0.0005, ****P<0.0001.

Trols irrespective of disease activity and previous treatment (median 10/μL for non-progressive, 36/μL for progressive untreated, 53/μL for progressive treated vs. 5/μL for controls; P=0.02, P=0.0002 and P<0.0001, respectively). Higher numbers of proliferating CD4+ T cells were observed also in progressive patients compared to controls, irrespective of previous treatment (median 75/μL for progressive untreated and 41/μL for progressive treated vs. 10/μL for controls; P=0.006 and P<0.001, respectively) (Figure 4C and D).

**Distribution of functional CD4+ T-helper subpopulations in relation to disease activity**

T-helper subpopulations were defined by CCR6 and CXCR3 expression as Th1 (CCR6/CXCR3+), Th2 (CCR6/CXCR3) and Th17 (CCR6/CXCR3) cells. Tregs were defined by the expression of CD4, CD25 and CCR4 and CD127low.22,23 Both non-progressive and progressive untreated CLL patients had higher numbers of Th1 cells compared to controls (median 406 and 1064 vs. 139/μL; P<0.0001 and P=0.001, respectively). However, progressive treated patients had lower Th1 numbers (median 62/μL) compared to both controls (P<0.009) and the other patient groups (P<0.0001) and the other patient groups (P<0.0001) and the other patient groups (P<0.0001 and P=0.0001 compared to non-progressive and progressive untreated, respectively). The number of Th1 cells was lower only in patients treated with cyclophosphamide/fludarabine compared to controls (P=0.01). Higher Th2 numbers were observed in non-progressive patients compared to controls (median 833 and 599/μL; P=0.03), but progressive pre-treated patients had lower numbers of Th2 cells compared to untreated (P<0.005). The numbers of Th17 cells were higher in progressive untreated patients compared to controls (median 196 and 109/μL; P=0.04) but progressive pre-treated patients had lower Th17 numbers compared to controls (P=0.03) and untreated patients (P=0.002 and P=0.003, for non-progressive and progressive untreated, respectively) (Figure 5A).

No difference was observed in the percentage of Tregs comparing CLL patients and controls (median 4.8% for...
non-progressive, 4.2% for progressive CLL patients and 4.2% for controls, respectively; \(P=0.5\) (Online Supplementary Table S2). However, the absolute number of Tregs was higher in untreated CLL patients compared to controls (median 72/mL for non-progressive and 78/mL for progressive untreated vs. 37/mL for controls; \(P=0.04\) and \(P=0.002\), respectively), while no difference was seen for progressive pre-treated patients (median 54/mL) (Figure 5B). Nevertheless, Tregs were higher in patients pre-treated with cyclophosphamide/fludarabine as well compared to controls (\(P=0.04\)). Low numbers of CD8+ cells expressing CD103 were observed in CLL patients, though higher in non-progressive (n=27) and progressive untreated (n=14) patients compared to progressive previously treated patients (n=10) (median 3/mL vs. 0.2/mL; \(P=0.006\) and \(P=0.002\), respectively).

Discussion

In the present study, we analyzed the T-cell phenotype focusing on immune checkpoints and activation markers in CLL patients with different clinical characteristics. Since the total T-cell numbers may vary considerably between CLL patients and healthy individuals, between patients in different phases of the disease, and depending on previous treatments, we chose to compare absolute cell numbers. Percentage numbers are reported in Online Supplementary Table S2.

Increased T-lymphocyte counts, as well as expansion of CD8+ and CD4+ T cells, have been described in CLL,\(^4\)\(^-\)\(^6\) with a relatively higher increase in CD8+ cells resulting in a low CD4/CD8 ratio compared to controls.\(^2\)\(^-\)\(^4\) We found that CLL patients irrespective of disease phase and previous treatment had significantly higher numbers of CD8+ cells compared to controls. There was no significant difference in the distribution of the CD4+ and CD8+ subsets within the CD8+ population between untreated patients and controls. Nevertheless, pre-treated patients had significantly higher numbers of CD8+ cells.

Several studies have investigated the expression of PD-1 and CTLA-4 in CLL patients, but the results are contradictory. An increase in PD-1+CD8+ T cells in CLL patients, particularly within the effector memory subset, was noted by Riches et al.,\(^3\) while Tonino et al.\(^2\) found that PD-1 expression was decreased. Brusa et al.\(^3\) found significantly higher PD-1 expression in CD4+ and CD8+ T cells from CLL patients, but could not identify any association of significance between PD-1 expression and disease stage, treatment requirements or unfavorable molecular or cytogenetic markers. Novak et al.\(^3\) recently reported higher numbers of PD-1-expressing T cells within both the CD4+ and CD8+ subsets in CLL patients but no significant difference between patients in different phases of the disease. Finally, an association between the PD-1/PD-L1 axis and T-cell dysfunction in progressive disease has been reported.\(^3\)\(^2\)\(^3\)

A comprehensive summary of the relative changes we observed in absolute numbers of T-cell subpopulations and T cells expressing immune checkpoints or activation/proliferation markers in different subgroups of CLL patients compared to healthy controls is provided in Figure 6. In contrast to a previous report,\(^3\) we observed that the absolute numbers of CD4+ cells expressing PD-1 were significantly increased only in CLL patients with progressive disease compared to controls. The difference was more marked for pre-treated patients. Within the CD8+ subset, only pre-treated patients had significantly higher numbers of PD-1+ expressing cells compared to controls. This observation may indicate that T cells in progressive patients display features of exhaustion, which seemed to be accentuated after treatment. Whether this may relate to the treatment per se or to the fact that previously treated patients have more advanced disease cannot be fully elucidated at present.

It is known that the distribution of memory T-cell subtypes is altered in CLL patients. The expression of PD-1 on CD4+ effector memory cells is considered to be a marker...
of chronic activation. We noted that CLL patients had higher absolute numbers of CD4+ effector memory cells expressing PD-1 compared to controls irrespective of disease phase and previous treatment. CD4+ central memory cells also displayed high PD-1 expression. This subset was expanded in CLL patients, but only in those untreated. Moreover, naïve CD4+ cells expressing PD-1 were significantly higher in untreated CLL patients compared to controls. Effector CD4+ cells were not expanded but showed a high PD-1 expression. Collectively, these data may indicate a persistent (chronic) antigen exposure in CLL patients inducing T-cell exhaustion in all the CD4+ subsets, preferentially those antigen-experienced (CD45RO−), i.e. central memory and effector memory cells.

High numbers of effector memory cells were observed in the CD8+ subset in all the patients, and significantly higher PD-1 expression was observed in progressive patients. Effector CD4+ cells were not expanded but showed a high PD-1 expression. Collectively, these data may indicate a persistent (chronic) antigen exposure in CLL patients inducing T-cell exhaustion in all the CD4+ subsets, preferentially those antigen-experienced (CD45RO−), i.e. central memory and effector memory cells.

Figure 4. CD69 and intracellular Ki67 expression in T cells from chronic lymphocytic leukemia (CLL) patients and controls. Absolute numbers of (A) CD69+CD4+, (B) CD69+CD8+, (C) Ki67+CD4+, (D) Ki67+CD8+ T cells from progressive (P) and non-progressive (NP) CLL patients and healthy controls. Box plots display cumulative data with line at median. Only significant statistical values are reported. *P<0.05, **P<0.005, ***P<0.0005, ****P<0.0001.
this, we observed no expression of CD137 on freshly isolated CLL T cells, but expression could be induced in both CD4+ and CD8+ cells by in vitro stimulation, in particular in progressive patients.

Chronic lymphocytic leukemia patients had higher numbers of Th1, Th2 and Th17 cells compared to controls. No significant difference between non-progressive and progressive patients was observed. This is in contrast to previous data based on cytokine production, showing increased secretion of IL-4 in CLL, suggested to be due to a Th2 polarization during disease progression.20,41,44 We observed that previously treated progressive patients had significantly lower numbers of all three subsets. Consistent with previous data, we found that absolute numbers of Tregs were higher in untreated CLL patients compared to controls, independent of disease phase, but lower in previously treated patients.

Finally, we confirmed that both CD4+ and CD8+ T cells in progressive CLL patients display an activated phenotype (CD69+), as also shown previously.6 Moreover CLL patients had significantly higher numbers of proliferating CD4+ and CD8+ T cells, which was more evident at disease progression.

Taken together, our results suggest that disease activity and previous treatment have a different impact on T-cell profile in CLL. The disease per se implies a number of changes in T cells (Table 2). At disease progression the most remarkable alteration occurring in the CD4+ subset is an increase in CD69+ cells, while in the CD8+ subset more extensive changes take place. In addition to higher numbers of CD69+ cells, within the CD8+ subset, higher numbers of proliferating (Ki67+), effector memory and effector cells were noted. However, PD-1 and CTLA-4 expression in progressive disease were so high that it is reasonable to assume that these cells have heavily impaired immune functions, as also suggested by previously published data.30,32 CLL treatment also seemed to dramatically affect T cells, in particular the CD4+ subset, in which a decrease...
of all T-helper subsets (Th1, Th2, Th17) was observed. A decrease in naïve T cells in both the CD4+ and the CD8+ subsets was also related to therapy. We tried to define more specifically the impact of different treatment regimens on T-cell phenotype by further subgrouping the patients into those who had received alemtuzumab and those who had received fludarabine/cyclophosphamide, since these drugs have a known effect on T cells.45,46

The number of Th1 cells was significantly lower while Tregs were higher in patients treated with cyclophosphamide/fludarabine compared to controls; intracellular CTLA-4 expression seemed to be affected by both pre-treatment with both alemtuzumab and cyclophosphamide.

Different treatments did not seem to have a different impact on the expression of immune checkpoints and activation markers. Overall, the IGHV mutational status seemed to have a minor impact. Unfortunately, we do not have cyogenetic data for all the patients, since in Sweden analysis by interphase fluorescence in situ hybridization is routinely performed only in patients requiring therapy.

Therapeutic interference with T-cell exhaustion by targeting co-stimulatory and inhibitory pathways may be beneficial to increase anti-tumor T-cell responses in CLL patients. In particular, immune checkpoint blockade with anti-PD1 mAb might be successful also in heavily pretreated chemo-refractory patients. Even though PD-1 blockade alone might not be enough to reanimate exhausted T cells in CLL,48 a combined approach either with targeted drugs or immunotherapies directed against different receptors might be a rewarding approach in this patient subgroup.

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