T cells in chronic lymphocytic leukemia display dysregulated expression of immune checkpoints and activation markers

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ABSTRACT

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 increased expression of immune checkpoints, abnormal subset distribution, and a higher proportion of proliferating cells compared to healthy T cells. Disease activity and previous treatment shape the T-cell profile of chronic lymphocytic leukemia patients in different ways.

Introduction

Chronic lymphocytic leukemia (CLL) patients have dysregulated immune functions resulting in impaired antitumor immunity and increased risk for infections. Profound defects in T-cell functions have been described as an imbalance of T-cell subsets,[1] defective immune synapse formation with antigen presenting cells,[2] impaired cytotoxic effector function[3] and high frequency of regulatory T cells (Tregs).[4-6]

A number of co-signaling receptors, known as immune checkpoints, participate in the regulation of T-cell-driven immune responses. CD28 is constitutively expressed on $CD4^+$ and $CD8^+$ T cells providing the primary co-stimulatory signal for T-cell activation.[7] Upon T-cell stimulation, a number of cell surface molecules belonging to the

tumor necrosis factor (TNF)-receptor family are up-regulated and deliver co-stimulatory signals. CD137 is such a molecule. It binds to its ligand (CD137L), a member of the TNF family, expressed on macrophages, activated B cells and dendritic cells (DCs) enhancing T-cell proliferation and cytolytic effector functions.[8] The CD28 homolog cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) is expressed on activated T cells and has an inhibitory role in regulating T-cell activation.[9]

Another negative co-stimulatory molecule is programmed death-1 (PD-1), expressed on activated $CD4^+$ and $CD8^+$ T cells, natural killer (NK) T cells, B cells, activated monocytes and DCs. Upon binding to the ligands PD-L1 and PD-L2, PD-1 inhibits T-cell functions reducing T-cell receptor signaling and target cell lysis.[10] Except for cells of the macrophage lineage, PD-L1 has low expression in normal tissues; on the other hand, it is highly expressed on various tumors and can be further enhanced by tumor environmental factors.[11,12] The PD-1/PD-L1 pathway is considered a central regulator of T-cell exhaustion, a condition characterized by deteriorated T-cell effector function due to chronic antigen stimulation.[13] Tumor cells as well as pathogens exploit these inhibitory signals to hamper immune eradication.

The aim of the present study was to evaluate the expression of the immune checkpoints CD137, CTLA-4 and PD-1 in CLL patients at different phases of the disease as well as the distribution of various $CD4^+$ and $CD8^+$ T-cell subsets. We aimed to provide a comprehensive analysis of T-cell phenotype in CLL and to discriminate between alterations that are due to the disease itself and disease activity and those related to treatment.

We show that diverse T-cell alterations are related to distinct clinical situations, i.e. disease activity and previous treatment. The PD-1 receptor expression was markedly increased in active disease, especially in heavily treated patients.

Methods

Patients

Peripheral blood samples from 80 CLL patients were collected at the Department of Hematology, Karolinska University Hospital, Stockholm, Sweden, as well as from 9 age- and sex-matched controls. Patients were grouped according to disease activity: non-progressive *versus* progressive (i.e. fulfilling criteria for active disease[14]). Characteristics of the patients and controls are shown in Table 1. Ninety percent of the patients were cytomegalovirus (CMV)-positive, in line with the prevalence in the Swedish population aged over 60 years.[15] The research project was approved by the regional ethics committee (*www.epn.se*) and conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from study participants.

Flow cytometric analysis of lymphocyte subsets on whole blood

Cells were washed after lysis of red blood cell, resuspended in Cell Staining Buffer (CSB) (BioLegend, San Diego, CA, USA) and stained with CD19-AF488, CD16+56-PE, CD4-PerCp, CD3-PE-Cy7, CD8-APC and CD45-AF700 (Bio-Legend). After incubation and washing, cells were resuspended in CSB and analyzed by FACSCanto II flow cytometer and the FACSDiva v.6.1.3 (BD Biosciences, San Diego, CA, USA) or FlowJo v.8.8.2 (TreeStar, Ashland, OR, USA) softwares.

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, CD3-PE-Cy7, -AF700 and -PerCP, CD8-APC and -AF700, CD45-AF700, CD5-PE and -PerCP, CD45RO-FITC, HLA-DR-PerCp, CD25-APC, CD45RA-AF488, PD-1 (CD279)-PE, CD69-AF488, CTLA-4 (CD152)-PE, Ki-67-AF647 (Bio-Legend), CCR6 (CD196)-PE, CCR4 (CD194)-PE, CD127-PE-Cy7, CXCR3 (CD183)-APC, 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.[16] 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.[17,18] 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.[19]

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. Further details are provided in the *Online Supplementary Appendix.*

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 (alemtuzumab or not; fludarabine/cyclophosphamide or not) and IGHV mutational status (*data not shown*).

Table 1. Characteristics of patients and controls at time of testing.

Controls (n=9)				
Sex	Male, n	6		
	Female, n	3		
Age	Years (median, range)	65 (45-79)		
Patients (n=80)				
Disease phase[a]		Non-progressive (n=39)	Progressive (n=41)	Total (n=80)
Sex	Male, n	21	29	48
	Female, n	18	12	30
Age	Years, median (range)	67 (49-82)	69 (47-85)	69 (47-85)
Modified Rai stage[a]	Low risk, n	28	2	29
	Intermediate risk, n	11	11	21
	High risk, n	0	28	28
IGHV status	Mutated, n	22*	17**	43
	Unmutated/subset #2, n	10	24	34
	Undefined, n	3	0	3
Previous treatment	No, n	39	22	60
	17p deletion/*TP53* mutation	0/4	2/22	
	Yes, n	0	19	18
	17p deletion/*TP53* mutation	0/0	7/19	
	Previous therapy			
	Chlorambucil		5	
	FC or FCR		10	
	BR		2	
	Alemtuzumab		7	
	Other		3	
	≥ 2 previous treatment lines		10	
Chemotherapy-refractory	No, n		13/19	
	Yes, n		6/19	
Chemotherapy-refractory and/or 17p deletion/*TP53* mutation			11/19	

[a]As defined by Hallek *et al.*[14] *Of which 3 borderline IGHV mutated (97%-98% identity). **Of which 2 borderline IGHV mutated. FC: fludarabine + cyclophosphamide; FCR: fludarabine + cyclophosphamide + rituximab; BR: bendamustine + rituximab.

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 1C).

No difference was seen with regard to CD8$^+$ T cells comparing patients and controls, with the exception of previously treated progressive patients, who had higher numbers of PD-1$^+$ CD8$^+$ T cells compared to controls and non-progressive patients (median 389 *vs.* 121 *vs.* 143/μL; *P*=0.001 and *P*=0.007, respectively) (Figure 1B). Subgrouping based on the IGHV mutational status showed that this was only observed in the unmutated group. A moderate positive correlation was also observed between PD-1$^+$CD8$^+$ T cells and total lymphocyte count (r=0.43, *P*<0.0001) (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 irrespective of disease activity and previous treatment. The frequency of central memory T cells was also higher in CLL patients compared to controls, but only in those untreated. Naïve T cells were dramatically reduced in pre-treated patients compared to controls and untreated. No difference was seen for effector T cells (Figure 2A).

Similarly, higher absolute numbers of CD8$^+$ effector memory and effector cells were found 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

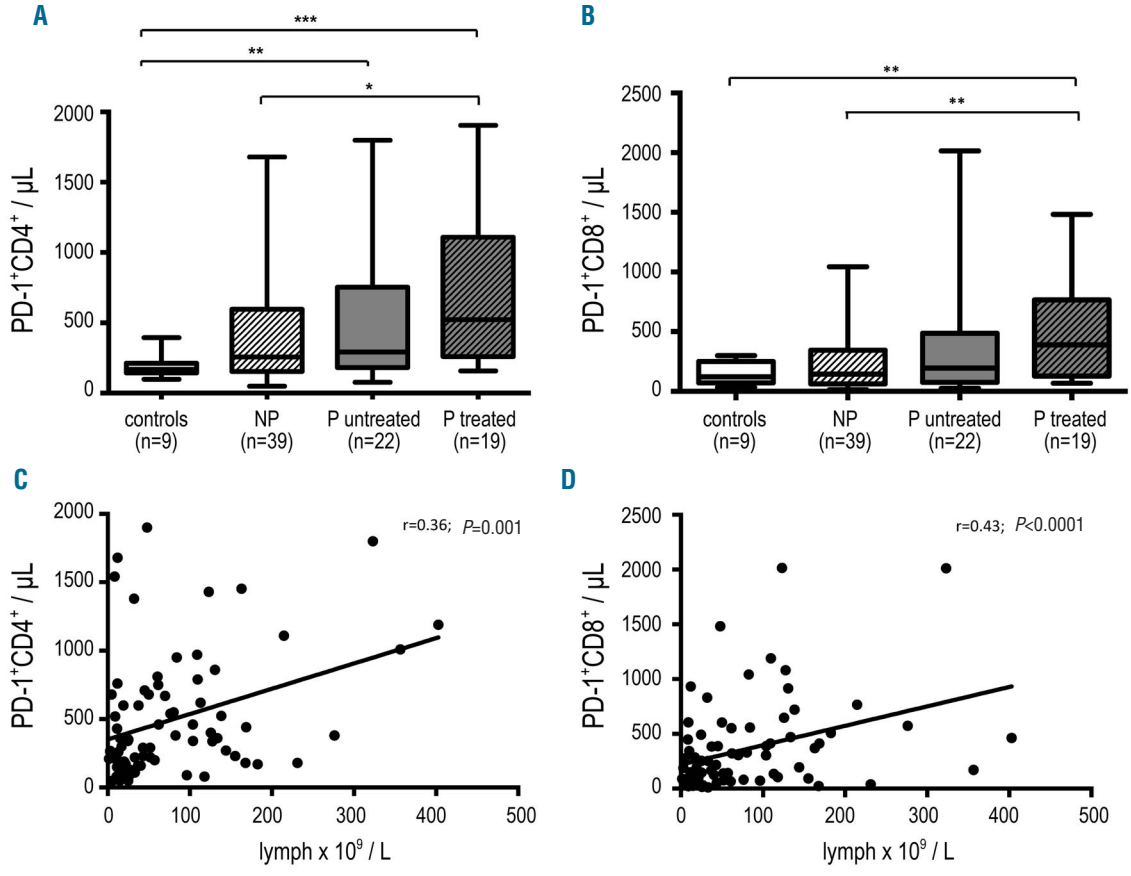

Figure 1. PD-1 expression in T cells from chronic lymphocytic leukemia (CLL) patients and controls. Absolute numbers of (A) PD-1$^+$CD4$^+$ and (B) PD-1$^+$CD8$^+$ T cells from progressive (P) and non-progressive (NP) CLL patients compared to controls. Box plots display cumulative data with line at median. *P<0.05, **P<0.005, ***P<0.0005. Correlation of (C) PD-1$^+$CD4$^+$ and (D) PD-1$^+$CD8$^+$ T cells with total lymphocyte count at the time of testing (n=80).

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 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<0.005) (Figure 3A). Numbers of CD4$^+$ 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

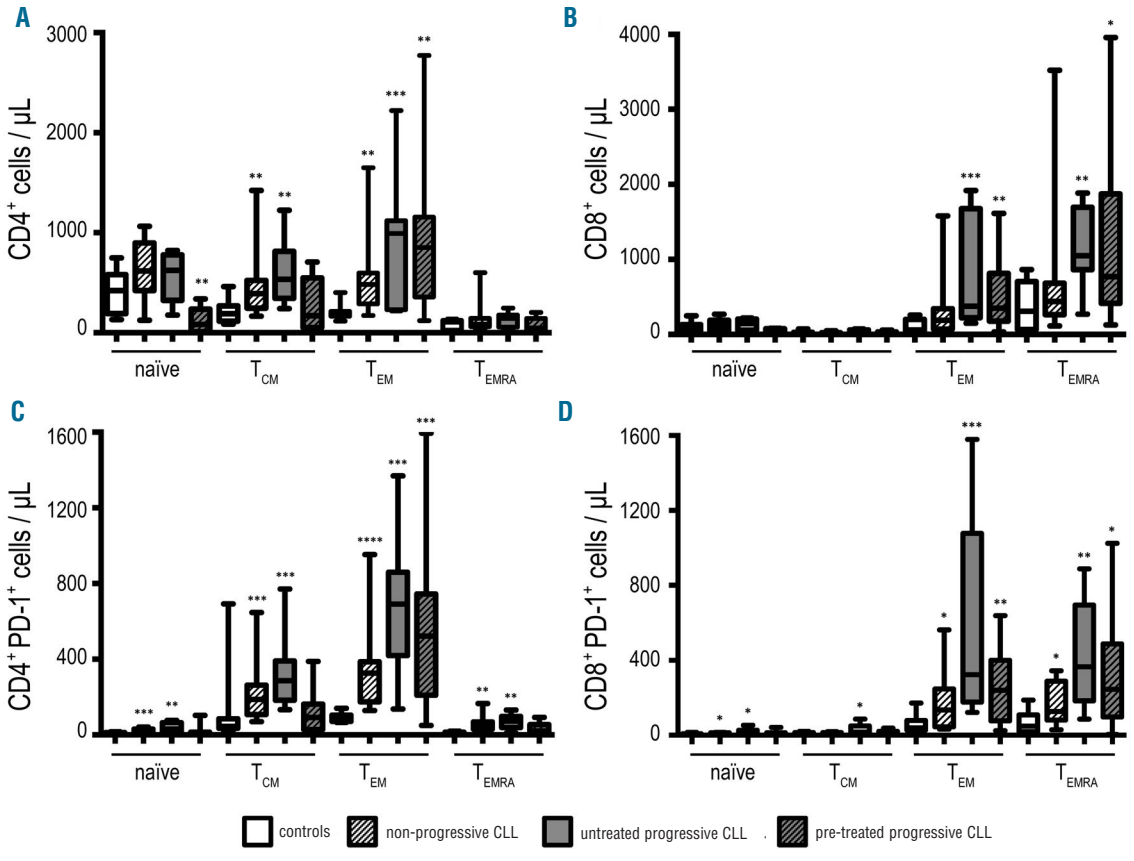

Figure 2. Comparative analysis of T-cell memory subsets in chronic lymphocytic leukemia (CLL) patients compared to controls. Absolute numbers of (A) $CD4^+$, (B) $CD8^+$, (C) $CD4^+PD\text{-}1^+$ and (D) $CD8^+PD\text{-}1^+$ naïve, central memory (CM), effector memory (EM) and effector (EMRA) cells in healthy controls (n=9) were compared to non-progressive (n=13), untreated progressive (n=8) and pre-treated progressive (n=12) CLL patients. 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.

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/µ; 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 % $CTLA\text{-}4^+CD4^+$ 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 $CD8^+$ Tregs[20] 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 P=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	Disease: non-progressive CLL vs. healthy controls	Effect of disease phase: progressive untreated vs. non-progressive	Treatment: progressive pre-treated vs. progressive untreated
$CD4^+$	↔	↔	↔
$PD1^+$	↔	↔	↔
T_{naive}	↔	↔	↓ (***)
T_{CM}	↑ (**)	↔	↓ (*)
T_{EM}	↑ (**)	↔	↔
T_{EMRA}	↔	↔	↔
i.c. $CTLA\text{-}4^+$	↑ (***)	↔	↔
$CD69^+$	↔	↑ (**)	↔
Th1	(****)	↔	↓ (***)
Th2	↑ (*)	↔	↓ (**)
Th17	↔	↔	↓ (**)
Tregs	↑ (*)	↔	↔
$Ki67^+$	↔	↔	↔

B	Disease: non-progressive CLL vs. healthy controls	Effect of disease phase: progressive untreated vs. non-progressive	Treatment: progressive pre-treated vs. progressive untreated
$CD8^+$	↔	↔	↔
$PD1^+$	↔	↔	↔
T_{naive}	↔	↔	↓ (**)
T_{CM}	↔	↔	↔
T_{EM}	↔	↑ (**)	↔
T_{EMRA}	↔	↑ (*)	↔
i.c. $CTLA\text{-}4^+$	↑ (****)	↑ (*)	↔
$CD69^+$	↓ (**)	↑ (**)	↔
$Ki67^+$	↑ (*)	↑ (*)	↔

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, 33/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 $CD127^{low}$.[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 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

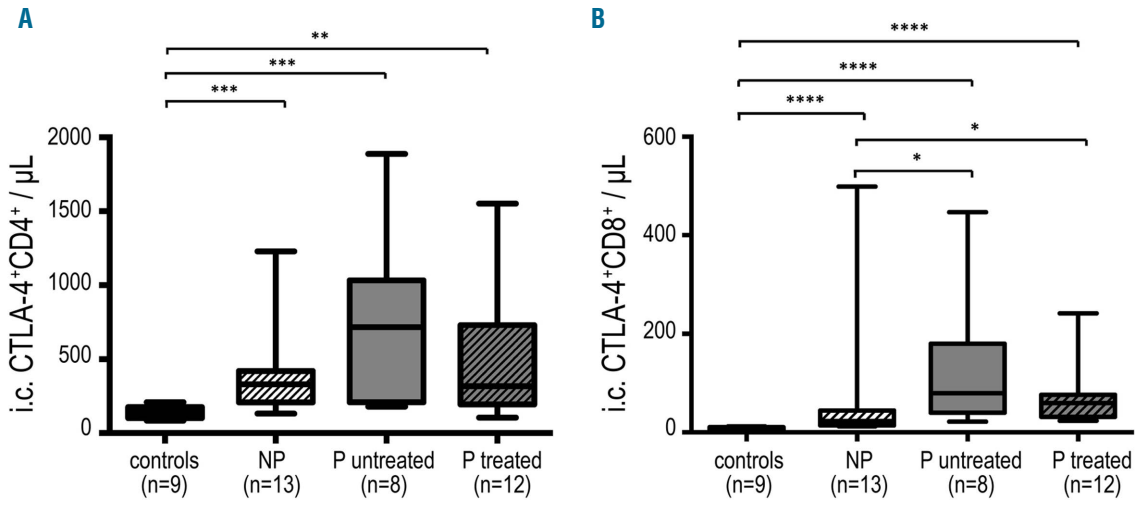

Figure 3. Intracellular CTLA-4 expression in T cells from chronic lymphocytic leukemia (CLL) patients and controls. Absolute numbers of (A) CD4+ and (B) CD8+ T cells with intracellular (i.c.) CTLA-4 expression in CLL patients and controls. Box plots display cumulative data with line at median. Only significant statistical values are reported. NP: non-progressive; P: progressive. *P<0.05, **P<0.005, ***P<0.0005, ****P<0.0001.

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,[24-26] with a relatively higher increase in CD8+ cells resulting in a low CD4/CD8 ratio compared to controls.[27,28] We found that CLL patients irrespective of disease phase and previous treatment had significantly higher numbers of CD3+ cells compared to controls. There was no significant difference in the distribution of the CD4+ and CD8+ subsets within the CD3+ 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.*[29] found that PD-1 expression was decreased. Brusa *et al.*[30] 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.*[31] 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.[32-34]

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,[31] 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 subsets is altered in CLL patients. The expression of PD-1 on CD4+ effector memory cells is considered to be a marker

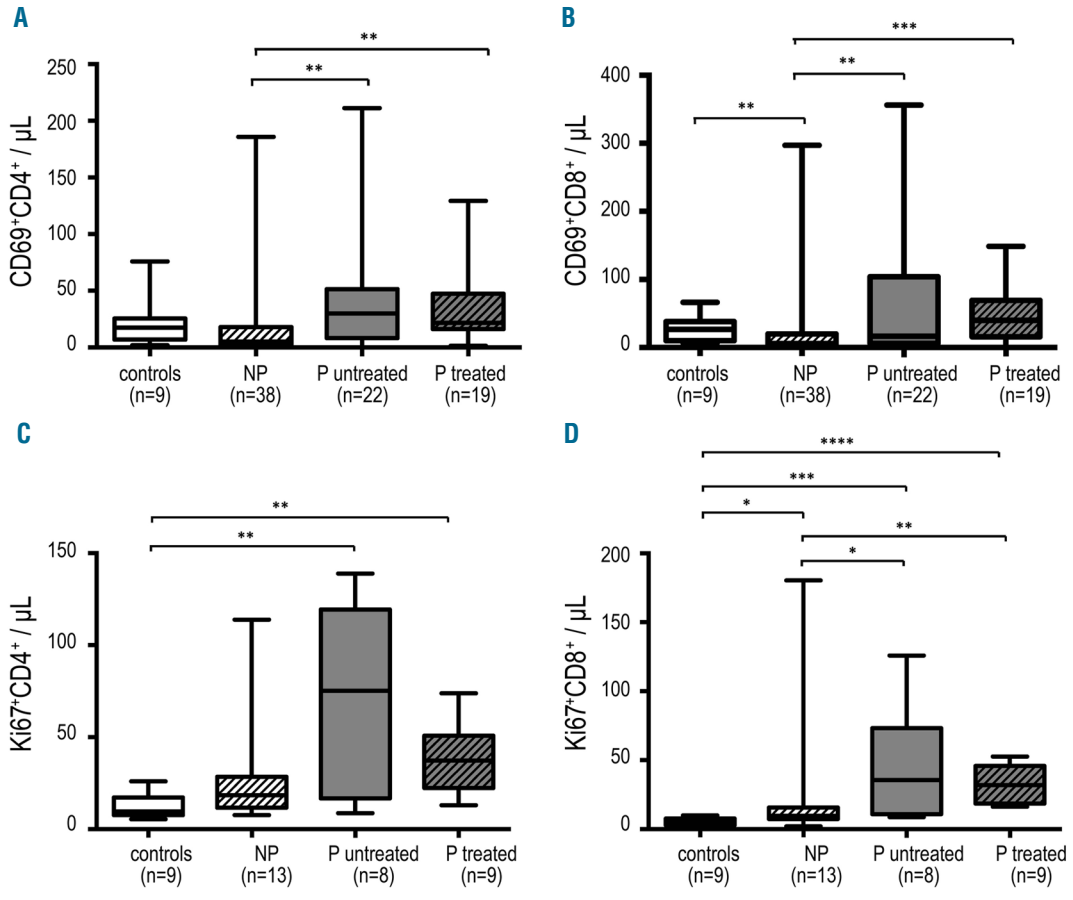

Figure 4. CD69 and intracellular Ki67 expression in T cells from chronic lymphocytic leukemia (CLL) patients and controls. Absolute numbers of (A) $CD69^+CD4^+$, (B) $CD69^+CD8^+$, (C) $Ki67^+CD4^+$, (D) $Ki67^+CD8^+$ T cells from progressive (P) and non-progressive (NP) CLL patients and healthy controls. 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.

of chronic activation.[35,36] 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. High numbers of PD-1^+ effector cells were also observed in all the patient subgroups. T cells from CLL patients display higher expression of inhibitory receptors, including PD-1, irrespective of CMV status.[3,32] Therefore, it is unlikely that the observed T-cell subset distribution is due to chronic stimulation by the CMV antigen, but is most likely due to stimulation by tumor antigens.

Contrary to previous reports,[30,33,37] we found no expression of PD-L1 on CLL cells. The reason for this is unclear, but might be due to the fact that we analyzed PD-L1 expression on freshly isolated cells, while other studies have analyzed the expression on purified $CD19^+$ cells.

One previous study reported that surface expression of CTLA-4 was decreased in CLL patients compared to controls,[38] while another study noted no difference[3] and three other studies[39-41] showed an increase. However, a significantly higher intracellular CTLA-4 expression in CLL T cells was found in all the previously published studies.[38,40,41] Moreover, Motta *et al.*[40] and Scrivener *et al.*[38] could not see any significant enhancement of CTLA-4 expression after *in vitro* stimulation, while Frydecka *et al.*[39] found that CTLA-4 expression increased over time in T cells but with a different kinetics to controls.

We noted no expression of surface CTLA-4 in either CLL patients or controls, but this could be induced in $CD4^+$ cells by *in vitro* stimulation, in particular in non-progressive patients. However, intracellular CTLA-4 expression was high in both $CD4^+$ and $CD8^+$ cells of CLL patients compared to controls. A hallmark of CTLA-4 is the trafficking to and from the plasma membrane following TCR stimulation.[9,42] CTLA-4 is engaged in the primary phase of T-cell activation, which might explain why chronically activated, exhausted T cells lack surface expression.

CD137 is poorly expressed or not at all in the resting T-cell state but up-regulated upon activation.[8] In line with

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.[25,43,44] We observed that previously treated progressive patients had significantly lower numbers of all three subsets. Consistent with previous data,[4,5] 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.[45] 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

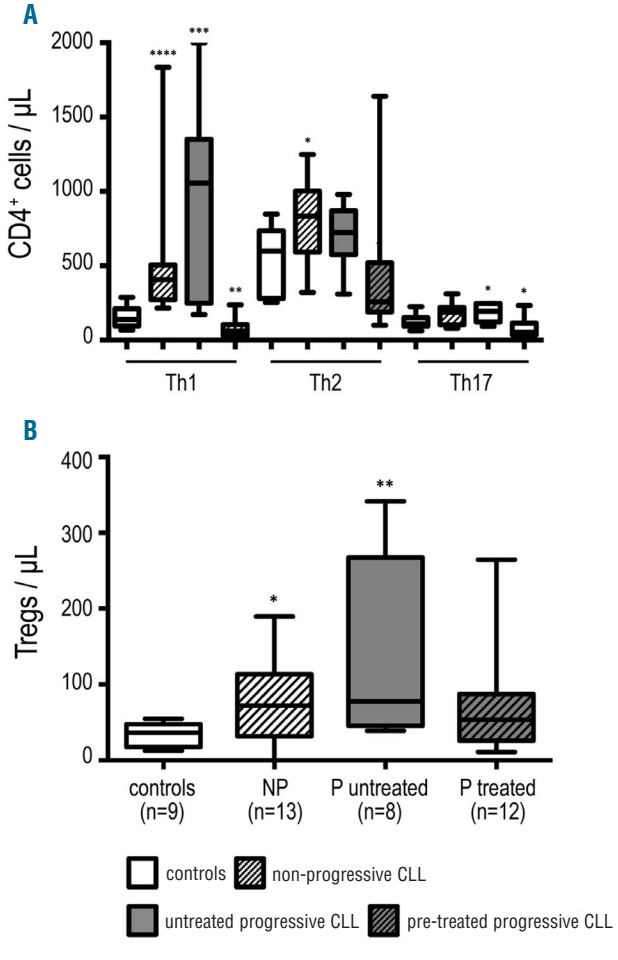

Figure 5. Comparative analysis of functional CD4+ T-helper subpopulations (Th1/Th2/Th17) and regulatory T cells (Tregs) in chronic lymphocytic leukemia (CLL) patients and controls. Absolute numbers of (A) Th1, Th2, Th17 and (B) Tregs cells in non-progressive (NP) (n=13), untreated progressive (P) (n=8), and pre-treated progressive (n=12) CLL patients compared with healthy controls (n=9). 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.

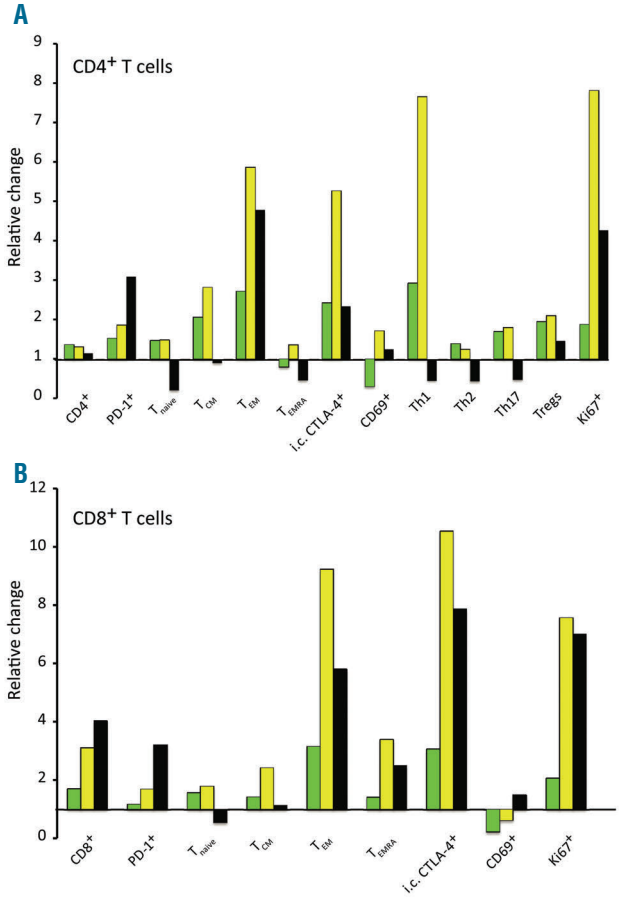

Figure 6. Relative change in absolute numbers of different T-cell subpopulations and T cells expressing immune checkpoints or activation / proliferation markers in chronic lymphocytic leukemia (CLL) patients compared to healthy controls. Relative change in the (A) CD4+ subset and (B) CD8+ subset calculated as median value patients/median value controls in non-progressive (green bars), untreated progressive (yellow bars) and pre-treated progressive (black bars) CLL patients.

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.[46,47]

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 pretreatment 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 cytogenetic 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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