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# Phenotypic complexity of T regulatory subsets in patients with B-chronic lymphocytic leukemia

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**Increased numbers of T regulatory ($T_{reg}$) cells are found in B-chronic lymphocytic leukemia, but the nature and function of these $T_{regs}$ remains unclear. Detailed characterization of the $T_{regs}$ in chronic lymphocytic leukemia has not been performed and the degree of heterogeneity of among these cells has not been studied to date. Using 15-color flow cytometry we show that $T_{reg}$ cells, defined using CD4, CD25, and forkhead box P3 (FOXP3), can be divided into multiple complex subsets based on markers used for naïve, memory, and effector delineation as well as markers of $T_{reg}$ activation. Furthermore FOXP3$^+$ cells can be identified among CD4$^+$CD25$^-$ as well as CD8$^+$CD4$^-$ populations in increased proportions in patients with chronic lymphocytic leukemia compared with healthy donors. Significantly different frequencies of naïve and effector $T_{regs}$ populations are found in healthy donor controls compared with donors with chronic lymphocytic leukemia. A population of CCR7$^+$CD39$^+$ $T_{regs}$ was significantly associated with chronic lymphocytic leukemia. This population demonstrated slightly reduced suppressive activity compared with total $T_{regs}$ or $T_{regs}$ of healthy donors. These data suggest that FOXP3-expressing cells, particularly in patients with chronic lymphocytic leukemia are much more complex for $T_{reg}$ sub-populations and transitions than previously reported. These findings demonstrate the complexity of regulation of T-cell responses in chronic lymphocytic leukemia and illustrate the use of high-dimensional analysis of cellular phenotypes in facilitating understanding of the intricacies of cellular immune responses and their dysregulation in cancer.**
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Chronic lymphocytic leukemia is one of the most common leukemias in adults in the Western hemisphere and is characterized by a slow, progressive accumulation of monoclonal B lymphocytes. Among patients with chronic lymphocytic leukemia, the progress of disease and response to therapy varies greatly for largely unknown reasons. Although chronic lymphocytic leukemia is a disease of B lymphocytes, a number of abnormalities in the T-lymphocyte compartment of the immune system have previously been described in patients, suggesting that an altered immune response occurs in response to the leukemia, or is a causal factor in the escape of the leukemic cells from normal immune recognition (Table 1).[1–8] Among the observed T-cell alterations is the intriguing finding of elevated frequencies of T regulatory ($T_{reg}$) cells in the peripheral circulation of patients with chronic lymphocytic leukemia.[5,7–10]

Natural $T_{reg}$ cells in humans are most often characterized as CD4$^+$ T cells with high expression of CD25 and the transcription factor forkhead box P3 (FOXP3), a master regulator of $T_{regs}$. $T_{regs}$ are also generally agreed upon to demonstrate a phenotype of CTLA-4$^+$, CD62L$^+$, CD127$^{low}$, and GITR$^+$.[11,12] In the peripheral circulation of healthy adults and elderly individuals, the majority of $T_{regs}$ express CD45RO, and are termed as effector $T_{regs}$, which

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**Table 1** T cell abnormalities associated with B-CLL in the literature

| *T-cell abnormality* | *References* |
|---|---|
| Increased CD8 population | Scrivener *et al*[1] |
| Decreased CD4:CD8 ratio | Reyes *et al*[2] |
| Increased CD4+CD45RO+ and CD8+ CD45RO+ | Dianzani *et al*[3] |
| Increased CD8+CD45RACD27− | Mackus *et al*[4] |
| Increased CD4+CD25+ $T_{regs}$ | Beyer *et al*[5] |
| Increased CD4+CD25+ FOXp3+ $T_{regs}$ | Giannopoulos *et al*[6] |
| Increased CD4+CD25+ CD127-FOXp3+ $T_{regs}$ | Jak *et al*[7] |
| CD4+CD25+ CD127-FOXp3+ $T_{regs}$ are CD45RO+ | Jak *et al*[7] |

Abbreviation: Treg, T-regulatory cell.

might reflect differentiation of either natural $T_{regs}$ derived from the thymus or induced $T_{regs}$ derived from memory cells in the peripheral circulation.[13–16] $CD45RA^+$ $T_{regs}$ predominate in cord blood, but persist into adulthood, where they represent a minority of $T_{regs}$ in the peripheral circulation.[17] The percentage of CD45RA expressing $T_{regs}$ decreases with increasing age in adults.[18] A number of other $T_{reg}$ subsets have been described by the expression of various markers. Some of these have been associated with increased $T_{reg}$ suppressive activity, including CD39, HLA-DR, and CD103.

Using $CD4^+CD25^{high}$ expression to identify $T_{regs}$, Beyer *et al*[5] found increased percentages of $T_{reg}$ in chronic lymphocytic leukemia compared with age-mismatched healthy donors, and noted a correlation between the frequency of $T_{regs}$ and stage of disease using the Binet staging classification. Giannopoulos *et al*[6,10] similarly noted an increase in the percentage of $T_{regs}$ in chronic lymphocytic leukemia using $CD4^+CD25^{high}$FOXP3 to identify $T_{regs}$. Although they also found a correlation between the frequency of $T_{regs}$ and Binet stage, they found no significant correlation with ZAP-70 or CD38. Using $CD4^+CD25^{bright}CD127^{low}$ to identify $T_{regs}$, Jak *et al*[7] found an increase in the absolute number of $T_{regs}$ in chronic lymphocytic leukemia and reported that the percentage of $T_{regs}$ increase with increasing stage of disease using the Rai classification. This group also reported a predominance of $CD45RO^+$ $T_{regs}$ in these patients, suggesting that these cells arise from the memory T-cell pool in a CD70-dependent manner and accumulate due to reduced apoptosis.[7] Recently, Weiss *et al*,[19] using $CD3^+CD4^+CD25^+CD127^-$ to identify $T_{regs}$, found a significant elevation of percentage of $T_{regs}$ in chronic lymphocytic leukemia compared with age-mismatched healthy control donors. This group further determined that $T_{regs}$ are an independent predictor of time to initial treatments in these patients. In the study by Weiss *et al*,[19] the percentage of $T_{regs}$ correlated with unmutated IgVH, high CD38 expression, as well as specific cytogenetic findings.

It is thought that $T_{regs}$ facilitate progression of disease in chronic lymphocytic leukemia, as well as in solid tumors, by suppressing T-cell anti-tumor responses.[20] In chronic lymphocytic leukemia, therapy with fludarabine, thalidomide, or lenalidomide has been reported to reduce the frequency, and limit the function, of $T_{regs}$ in these patients,[5,6] suggesting the efficacy of these therapies might be due at least in part to effects on $T_{regs}$. Chronic lymphocytic leukemia is also strongly associated with a progressive immunodeficiency in these patients and it has also been speculated that $T_{regs}$ arising in this disease might be a cause of the autoimmune complications.[21] $T_{regs}$ found in chronic lymphocytic leukemia patients neither have been characterized in any great detail nor have definitive reasons for their increased frequency in chronic lymphocytic leukemia been delineated.

In the current study, high-resolution phenotyping of T-cell subsets and characterization of $T_{reg}$ subpopulations in the circulation of chronic lymphocytic leukemia was accomplished using the 15-color immunophenotyping that included markers to identify $T_{regs}$ as well as those reported to be associated with naïve, memory, and suppressive activity in T cells. This permits more rigorous examination of $T_{regs}$ to discern complex phenotypes that may differ from those of healthy donors or that may correlate with progressive disease or the mutational status of the malignant population in chronic lymphocytic leukemia. Using probability state modeling (PSM)[22–24] these complex $T_{reg}$ phenotypes were then analyzed to better understand marker correlations defined by the high-dimensional data. A selected $T_{reg}$ subset identified in this manner was sorted and assayed for suppression of autologous T-cell responses.

## Materials and methods

### Patient Samples

Peripheral blood specimens from 21 patients with B-chronic lymphoid leukemia and 16 age-matched healthy donors were studied. Peripheral blood from healthy donors enrolled in the NHLBI study 09-H-0201 and 09-H-0229 was collected into vacutainer tubes containing sodium heparin. Written informed consent for all procedures and research collections was obtained. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation over Lymphocyte Separation Medium (LSM, ICN Biomedicals, Aurora, OH). Cells were viably frozen and kept in fetal calf serum containing 10% dimethyl sulfoxide and stored in liquid nitrogen. Peripheral blood was collected from treatment naïve chronic lymphocytic leukemia patients with RAI scores of 2 or less enrolled in the NCI study 97-C-0178 (ClinicalTrials.gov Identifier: NCT00019370) (Table 2). Mononuclear cells were isolated and viably frozen in a similar manner to the healthy donor cells. For all chronic lymphocytic leukemia patients, data were collected on whether the tumor cells expressed a mutated (m-chronic lymphocytic

leukemia) or an unmutated IgVH gene (u-chronic lymphocytic leukemia), as well as expression of CD38 and ZAP-70, to serve as markers of a more indolent disease and longer overall survival.[25,26]

### Flow Cytometry

Single-cell suspensions from viably cryopreserved samples were thawed and washed once in PBS. After washing, cells were incubated for 30 min in presence of LIVE/DEAD Aqua fixable viability dye (Invitrogen, Carlsbad, CA), followed by a wash in staining buffer (PBS supplemented with 2% normal mouse serum (Gemini Bioproducts, West Sacramento, CA)). To determine the proportion of $T_{regs}$, we stained the cells with a 13-color combination of the following monoclonal antibodies: anti-$CD3_{APC-Cy7}$, anti-$CD4_{V450}$, anti-$CD38_{PerCP-Cy5.5}$, anti-$CD25_{PE-Cy-7}$, anti-$CD127_{APC}$ (Becton Dickinson, San Jose, CA), anti-$CD45_{Qdot800}$, anti-$CD8_{Qdot605}$, anti-$CD27_{Qdot655}$, anti-HLA-$DR_{PE-Cy5.5}$, anti-$CD39_{FITC}$, anti-$CD103_{PE-Cy5}$ (Invitrogen, Eugene, OR), anti-CD45-$RA_{PE-TR}$ (Beckman Coulter, Miami, FL), anti-$CCR7_{Alexafluor700}$ (R&D Systems, Minneapolis, MN). After cell surface staining, the cells were permeabilized with eBioperm buffer (eBioscience, San Diego, CA) and stained with anti-FOXP3 $_{PE}$ (eBioscience, clone PCH101). After staining, cells were washed, fixed in PBS containing 2% formaldehyde, and acquired on an LSRII flow cytometer equipped with 405, 488, 532, and 638 laser lines using DIVA 6.1.2 software (Becton Dickinson). Data were analyzed with the FlowJo software version 8.8.6 (Treestar, San Carlos, CA) and by the GemStone probability state modeling software (Verity Software House, Topsham, ME). Lymphocytes were identified according to their light-scattering properties (Figure 1a) and then analyzed gated on viable cells and $CD45^+$ events. In $CD4^+$ and $CD8^+$ cells, four subpopulations could be defined, according to a consensus among several publications,[27–29] as follows: naïve cells ($CD45RA^+$), central memory (CM) cells ($CD45RA^-CD27^+CCR7^+$), effector memory (EM) cells ($CD45RA^-CD27^+CCR7^-$), and effector cells (EC) ($CD45RA^-CD27^-CCR7^-$). Conventional $T_{reg}$ cells were defined as viable $CD45^+CD3^+CD8^-CD4^+CD25^+FOXP3^+$ mononuclear cells (Figure 1a). Among the $T_{reg}$ population, as previously performed for CD4 and CD8 cells, we identified naïve $T_{reg}$ ($CD45RA^+$), CM $T_{reg}$ ($CD45RA^-CD27^+CCR7^+$), EM $T_{reg}$ ($CD45RA^-CD27^+CCR7^-$), and effector $T_{reg}$ ($CD45RA^-CD27^-CCR7^-$).

**Table 2** Patients characteristics

| | *Sex* | | *IgVH status* | | *CD38-Zap 70* | |
|---|---|---|---|---|---|---|
| *Median age (years)* | *Female* (%) | *Male* (%) | *uCLL* (%) | *mCLL* (%) | *Negative* (%) | *Positive* (%) |
| *HD* | | | | | | |
| 34.5 (20–56) | 40 | 60 | n.a | n.a | n.a | n.a |
| *B-CLL* | | | | | | |
| 66.4 (35–78) | 38 | 62 | 47.6 | 52.4 | 38.9 | 42.8 |
| | | | | | 19% unknown | |

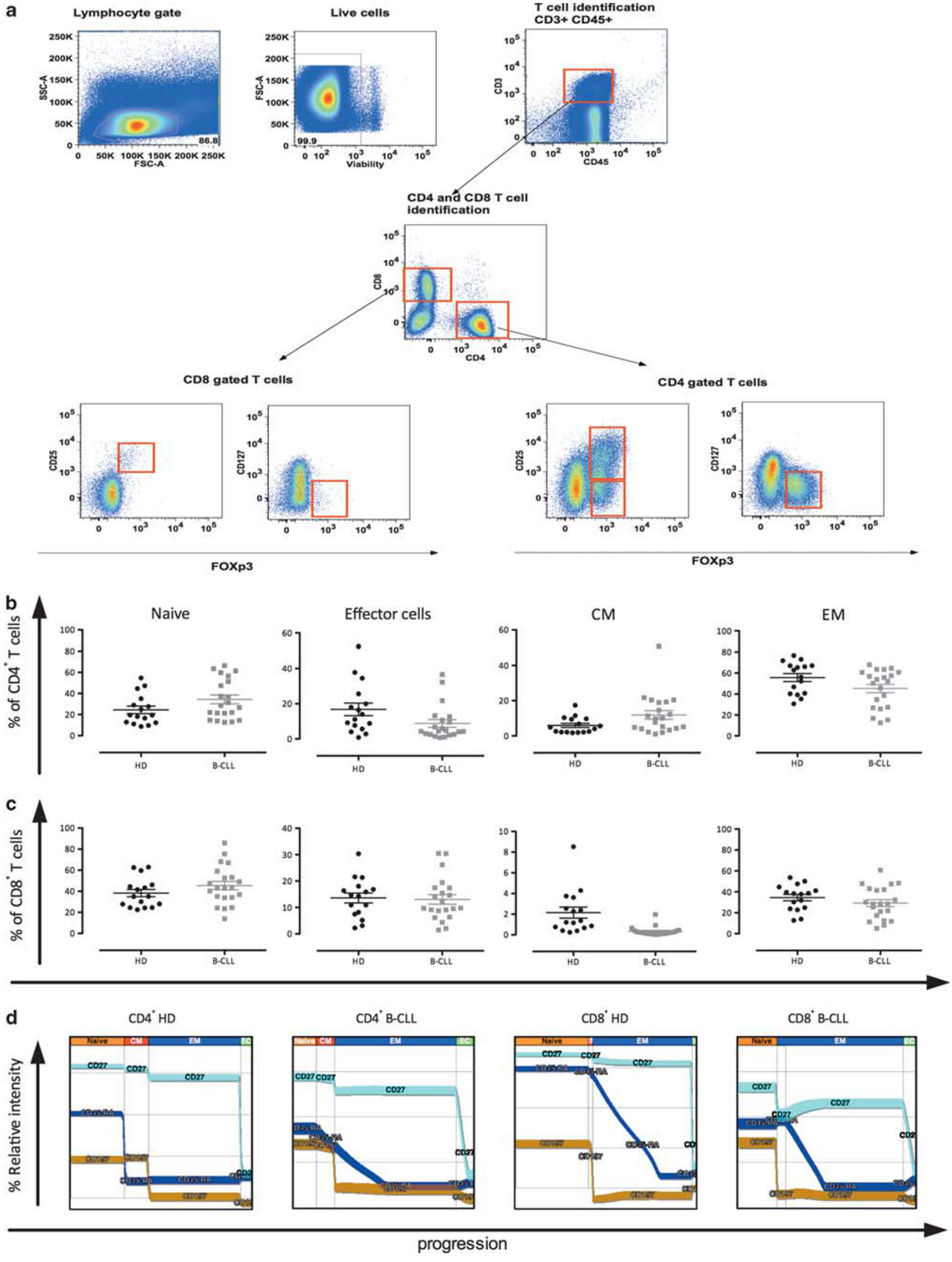
### Cell Sorting of $T_{regs}$

PBMCs were isolated by density gradient centrifugation over LSM. Cells were stained with the following antibodies: CD3, CD4, CD25, CCR7, and CD39. According to Baecher-Allan *et al*,[30,31] $CD4^+CD25^{high}$ is an appropriate gate for sorting viable $T^{regs}$ without using FOXP3 staining. Three populations were sorted using a FACS Aria flow cytometer (Becton Dickinson): $CD4^+CD25^-$ ($T_{effec}$), $CD4^+CD25^{high}$ ($T_{reg}$), and $CD4^+CD25^{high}CD39^+CCR7^+$ ($T_{reg}$DP) (Figure 3c). Subsequently, autologous $CD3^-$HLA-$DR^+$ cells were sorted from gamma-irradiated PBMCs (4000 Rad) to use as antigen-presenting cells (APCs).

### CFSE Labeling and $T_{reg}$ Suppression Assay

$T_{reg}$-mediated suppression assay was performed accordingly to Venken *et al*.[32] $CD4^+CD25^-$ cells

**Figure 1** Gating strategy to identify T regulatory ($T_{regs}$) population in chronic lymphocytic leukemia patients and CD4 and CD8 characterization. Single-cell suspensions from chronic lymphocytic leukemia were stained with a combination of 14 antibodies and a viable dye as described in Materials and methods. (**a**) Lymphocytes were identified based on their forward- and side-scatter properties. Subsequently, dead cells were excluded through the use of a viability dye. CD45 and CD3 were used to identify T cells ($CD45^+CD3^+$) among the previously selected viable lymphocytes. CD4 T cells and CD8 T cells were identified as uniquely expressing CD4 or CD8 antigens. Conventional $T_{regs}$ were defined as CD4 T cells co-expressing CD25 and transcription factor forkhead box P3 (FOXP3). CD127 expression was measured in FOXP3-expressing cells. $CD25^-$ $T_{regs}$ were defined as $CD4^+FOXP3^+CD25^-$ T cells. CD8 $T_{regs}$ were defined as $CD8^+CD25^+FOXP3^+$ T cells. In addition to $T_{regs}$ memory and naïve compartment of CD4 and CD8 was described. The graphs display the percentages (the mean percentage values ± s.e.m. are indicated by the bars) of naïve *vs* memory sub-population CD4 T cells (**b**) and CD8 T cells (**c**) in healthy donors (black circle) and chronic lymphocytic leukemia (gray square). (**d**) A probability state model constructed with GemStone (Verity Software House) was applied to CD4 T cells and CD8 T cells from healthy donors and from chronic lymphocytic leukemia. For these displays, the data were gated on light scatter, viability, and expression of CD45, CD3, and either CD4 or CD8 as described earlier. The *y*-axis is relative fluorescence intensity, whereas the *x*-axis displays staged progression from naïve to memory (CM, EM) and effector (EC) compartments. The width of each stage (naïve, EM, CM, EC) indicates the percentage of cells falling into these categories. The width of the bands corresponds to the variability of the data as the cells progress. These graphs reveal the same proportions of memory and naïve subsets as in the previous figures, although here it is possible to visualize relative intensity of marker expression along with putative transitions among these populations.


a
Lymphocyte gate
Live cells
T cell identification
CD3+ CD45+
CD4 and CD8 T cell identification
CD8 gated T cells
CD4 gated T cells
FOXp3
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% of CD4+ T cells
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Effector cells
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CD4+ HD
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CD8+ HD
CD8+ B-CLL
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progression

($T_{effec}$) were labeled using 2.5 μM CFSE (Cell trace CFSE cell proliferation kit, #C34554, Invitrogen) in PBS for 10 min at 37 °C. Cells were subsequently washed with RPMI supplemented with 10% heat-inactivated human AB serum (Sigma-Aldrich, St Louis, MO) and 1 × antibiotic and anti-mycotic solution (Invitrogen). For the suppression assay, 50 000 $T_{effec}$ cells were incubated with 50 000 APCs and differing amounts (where $T_{effec}$:$T_{reg}$ = 1:0, 1:1, 1:0.2) of various sorted $T_{reg}$ cell subsets in a 96-well flat bottom cell culture plate. Cultures were stimulated with mouse anti-human-CD3 antibody (Clone Okt-3, eBioscience) for 72 h at 37 °C in a humidified $CO_2$ chamber. Dilution of CFSE stain was read using an LSRII Flow cytometer (BD Biosciences) and data were analyzed using the FlowJo software (Treestar). Suppressive capacity of $T_{reg}$ toward $T_{effec}$ cells was expressed as the relative inhibition of the percentage of CFSE-low cells according to the following formula; $[100 \times (1-(y/x))]$, where $x$ = percentage of CFSE-low cells in $T_{effec}$ alone culture and $y$ = percentage of CFSE-low cells in $T_{effec}$:$T_{reg}$ culture[32] (Figure 3d).

### Statistical Analysis

Data obtained with cells from one donor were considered as one experiment ($n$). Statistical analysis performed on the results included the calculation of mean, s.e., and $P$-values by use of a multiple comparison test (two-way ANOVA test) or Mann–Whitney test (non-parametric $t$-test). Correlations between parameters were calculated using the Spearman's correlation, and Spearman's $r$ factor was reported. The significance level was set at $P = 0.05$, and the $P$-values were given for each series of experiments.

## Results

### Assessment of $CD4^+$ and $CD8^+$ T-Cell Subsets

The initial analysis of these data was carried out to determine whether there were differences in the frequencies of $CD8^+$ and $CD4^+$ T cells in patients with chronic lymphocytic leukemia compared with healthy donors. Among T cells, the percentage of $CD4^+$ cells was slightly lower in chronic lymphocytic leukemia patients compared with healthy donors but did not reach statistical significance: 47.4 ± 3.8% *vs* 58.5 ± 3.9% ($P = 0.08$). The percentage of $CD8^+$ T cells in chronic lymphocytic leukemia and healthy donors was similar: 31.4 ± 1.3% in chronic lymphocytic leukemia *vs* 36.1 ± 3.7% in healthy donors ($P = 0.2$). The CD4 to CD8 ratio was similar in both groups: 2.23 ± 0.4 in chronic lymphocytic leukemia and 2.1 ± 0.2 in healthy donors ($P = 0.84$; data not shown). Double-negative $CD4^-CD8^-$ T cells were significantly increased in the chronic lymphocytic leukemia; 19.6 ± 11% compared with 4.3 ± 3.6% in healthy donors ($P < 0.0001$).

Among $CD4^+$ and $CD8^+$ T cells, the proportions of naïve, EC, CM and EM cells (as described in Materials and methods) were measured (Figures 1b–d). The frequency of naïve $CD4^+$ population was increased in chronic lymphocytic leukemia, representing 34.4 ± 4.1% *vs* 24.4 ± 3.6% in healthy donors ($P = 0.06$; Figure 1b), whereas the proportion of naïve $CD8^+$ was unchanged: 45.4 ± 4% in chronic lymphocytic leukemia *vs* 38.2 ± 3.5% in healthy donors ($P = 0.27$; Figure 1c). A lower frequency of $CD4^+$ ECs was observed in blood from chronic lymphocytic leukemia patients compared with healthy donors (8.8 ± 2.1% *vs* 16.8 ± 3.6%, $P = 0.03$), but the proportion of $CD8^+$ ECs was unchanged. By contrast, the frequency of CM $CD4^+$ T cells was higher in chronic lymphocytic leukemia compared with healthy donors (11.8 ± 2.4% *vs* 5.9 ± 1.1%, $P = 0.05$) and the frequency of CM $CD8^+$ T cells was lower in chronic lymphocytic leukemia compared with healthy donors (0.3 ± 0.09% *vs* 2.2 ± 0.5%, $P = 0.0001$; Figure 1c). Among both $CD8^+$ T cells and $CD4^+$ T cells, the proportion of EM cells was unchanged.

### $T_{regs}$ and $T_{reg}$ Memory and Naïve Sub-populations

The percentage of natural (viable $CD45^+CD3^+CD8^-CD4^+CD25^{high}FOXP3^+$) $T_{reg}$ cells was significantly higher in chronic lymphocytic leukemia patients compared with healthy donors (12.8 ± 1.3% *vs* 5.4 ± 0.4%, $P < 0.0001$; Figure 2a). Flow cytometric analysis confirmed that the majority of $T_{regs}$ in healthy donors was $CD45RA^-$ (90.6 ± 1.7%), whereas in chronic lymphocytic leukemia patients, $T_{reg}$ cells were found in both $CD45RA^-$ and $CD45RA^+$ populations. In chronic lymphocytic leukemia, naïve $T_{reg}$ cells were significantly expanded compared with healthy donors (43.5 ± 4.7% *vs* 11.9 ± 1.4%, $P < 0.0001$; Figure 2b). Among the $CD45RA^-$ populations, $T_{reg}$ cells identified as EM ($CD45RA^-CCR7^-CD27^+$) were decreased in chronic lymphocytic leukemia compared with healthy donors (37.4 ± 4.4% *vs* 77.7 ± 1.2%, $P < 0.0001$; Figure 2c). The frequency of CM ($CD45RA^-CCR7^+CD27^+$) $T_{reg}$ was unchanged (8.6 ± 1.3% in chronic lymphocytic leukemia *vs* 5 ± 0.7% in healthy donors, $P = 0.62$; Figure 2d). The proportion of effector $T_{reg}$ cells was reduced in chronic lymphocytic leukemia compared with healthy donors (1.7 ± 0.4% *vs* 7.6 ± 1.1%, $P < 0.0001$; Figure 2e).

### High-Resolution Immunophenotyping of $T_{reg}$ Cells

To further characterize the phenotype of natural (viable $CD45^+CD3^+CD8^-CD4^+CD25^{high}FOXP3^+$) $T_{reg}$ cells present in chronic lymphocytic leukemia

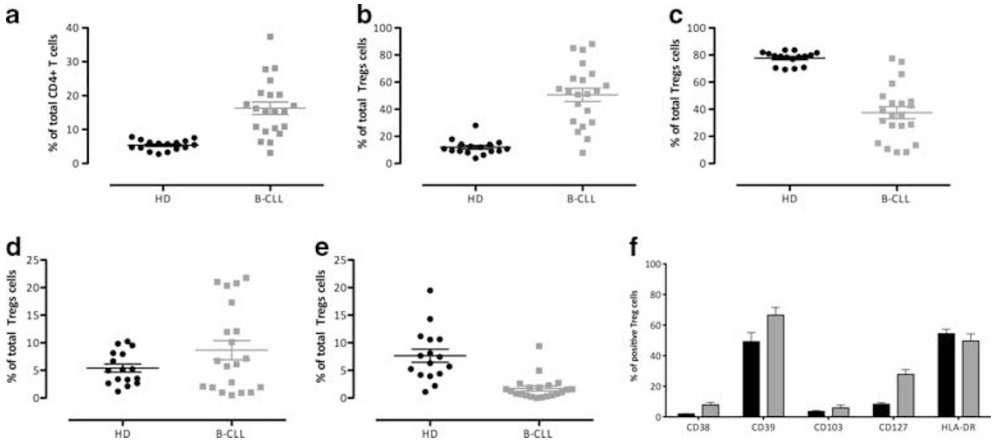


**Figure 2** Conventional T regulatory ($T_{regs}$), memory *vs* naïve subtypes and expression of activation markers in healthy donors and chronic lymphocytic leukemia. Cells were stained as described in Materials and methods and analysis was performed to identify conventional $T_{regs}$. (**a**) Percentages and the mean percentage values (±s.e.m.) of CD4 $T_{regs}$ in healthy donors (black) and chronic lymphocytic leukemia (gray). (**b**) Percentages and the mean percentage values (±s.e.m.) of naïve $T_{regs}$ in healthy donors (black) and chronic lymphocytic leukemia (gray) identified as $CD45RA^+$ cells among $T_{regs}$. (**c**) Percentages and the mean percentage values (±s.e.m.) of effector memory (EM) $T_{regs}$ identified as $CD27^+CCR7^-$ cells among $T_{regs}$ in healthy donors (black) and chronic lymphocytic leukemia (gray). (**d**) Percentages and the mean percentage values (±s.e.m.) of central memory (CM) $T_{regs}$ identified as $CD27^+CCR7^+$ cells among $T_{regs}$ in healthy donors (black) and chronic lymphocytic leukemia (gray). (**e**) Percentages and the mean percentage values (±s.e.m.) of effector $T_{regs}$ identified as $CD27^-CCR7^-$ cells among $T_{regs}$ in healthy donors (black) and chronic lymphocytic leukemia (gray). (**f**) Percentages and the mean percentage values (±s.e.m.) of $T_{regs}$ expressing activation markers CD38, CD39, CD103, CD127, and HLA-DR were measured in healthy donors (black) and chronic lymphocytic leukemia (gray).

and healthy donors, the cell surface expressions of HLA-DR, CD38, CD103, and CD39 were measured (Figure 2f). The frequencies of $T_{regs}$ expressing CD38, HLA-DR, or CD103 were not significantly different between healthy donors and chronic lymphocytic leukemia. Nevertheless, another activation marker, CD39, was increased in $T_{regs}$ from chronic lymphocytic leukemia patients compared with healthy donors (66.5±5% and 49.1±5.9%, respectively, $P=0.01$). In healthy donors, natural $T_{regs}$ are $CD127^{low}$ (Figure 1b),[11] but CD127 expression in chronic lymphocytic leukemia $T_{regs}$ was increased (27.8±3.1% in chronic lymphocytic leukemia *vs* 8.2±1.1% in healthy donors, $P<0.0001$).

In addition to examining expression of activation markers in the overall $T_{reg}$ population, a combination of these activation markers was examined. In chronic lymphocytic leukemia, a significant increase of the double-positive $CD39^+$ $CCR7^+$ $T_{reg}$ population was observed compared with healthy donors (37.8±5% and 4.1±0.7%, respectively, $P<0.001$). Consequently, the double-negative $T_{reg}$ population was lower in chronic lymphocytic leukemia than in healthy donors (32.9±4.3% and 48.8±5.6%, respectively, $P<0.001$). Figure 3 shows representative dot plots of the expression of CD39 *vs* CCR7 in a healthy donor (Figure 3a) and in a chronic lymphocytic leukemia (Figure 3b). In chronic lymphocytic leukemia, the dual-positive $CD39^+CCR7^+$ $T_{regs}$ were primarily $CD45RA^+$: 86.5±3.5% compared with 5.9±2% in healthy donors ($P<0.001$).

The same activation markers were studied among the memory/naïve $T_{reg}$ sub-populations (Table 3). A different pattern of activation was observed among the $T_{reg}$ in chronic lymphocytic leukemia patients: naïve $T_{reg}$ expressed more CD38, CD39, CD127, and HLA-DR compared with healthy donors (two- to threefold increase, $P<0.0001$). In the CM $T_{reg}$ sub-population, HLA-DR expression was significantly decreased in chronic lymphocytic leukemia patients ($P<0.0001$). In the EM $T_{reg}$ sub-population, CD127 and HLA-DR expression was lower in chronic lymphocytic leukemia patients compared with healthy donors; conversely, CD38 expression was higher in chronic lymphocytic leukemia compared with healthy donors. Finally, in the effector $T_{reg}$ sub-population, HLA-DR expression was significantly decreased in chronic lymphocytic leukemia patients ($P=0.006$), and CD38 was also decreased ($P=0.003$).

## Analysis of $T_{reg}$-Mediated Suppression of Polyclonal T-Cell Responses

To evaluate the *in vitro* suppressive capacity of $T_{regs}$ toward responder $T_{effec}$ cells, $CD4^+CD25^{high}$ T cells ($T_{reg}$) and $CD4^+CD25^-$ T cells ($T_{effec}$) were sorted from seven healthy donors and five chronic lymphocytic leukemia patients (Figure 3c). Subsequently, the sorted cells were used to setup an autologous coculture assay as described in Venken *et al.*[32] Fifty thousand CFSE-labeled $CD4^+$ CD25 T cells (responder cells ($T_{effec}$)) were cocultured with varying amounts of $CD4^+CD25^+$ T cells ($T_{reg}$; ratio 1:0, 1:0.2, and 1:1) and stimulated with soluble anti-CD3 antibody in the presence of $10^5$ irradiated autologous $CD3^-HLA\text{-}DR^+$PBMCs.

To determine the undivided peak fluorescence (Figure 3d), CFSE-labeled $CD4^+CD25$ T cells ($T_{effec}$) were also cultured in the absence of anti-CD3 antibody (Figure 3d, unstimulated). Cells were subsequently analyzed for CFSE by flow cytometry. In healthy donors, $CD4^+CD25^+$ $T_{reg}$ cells

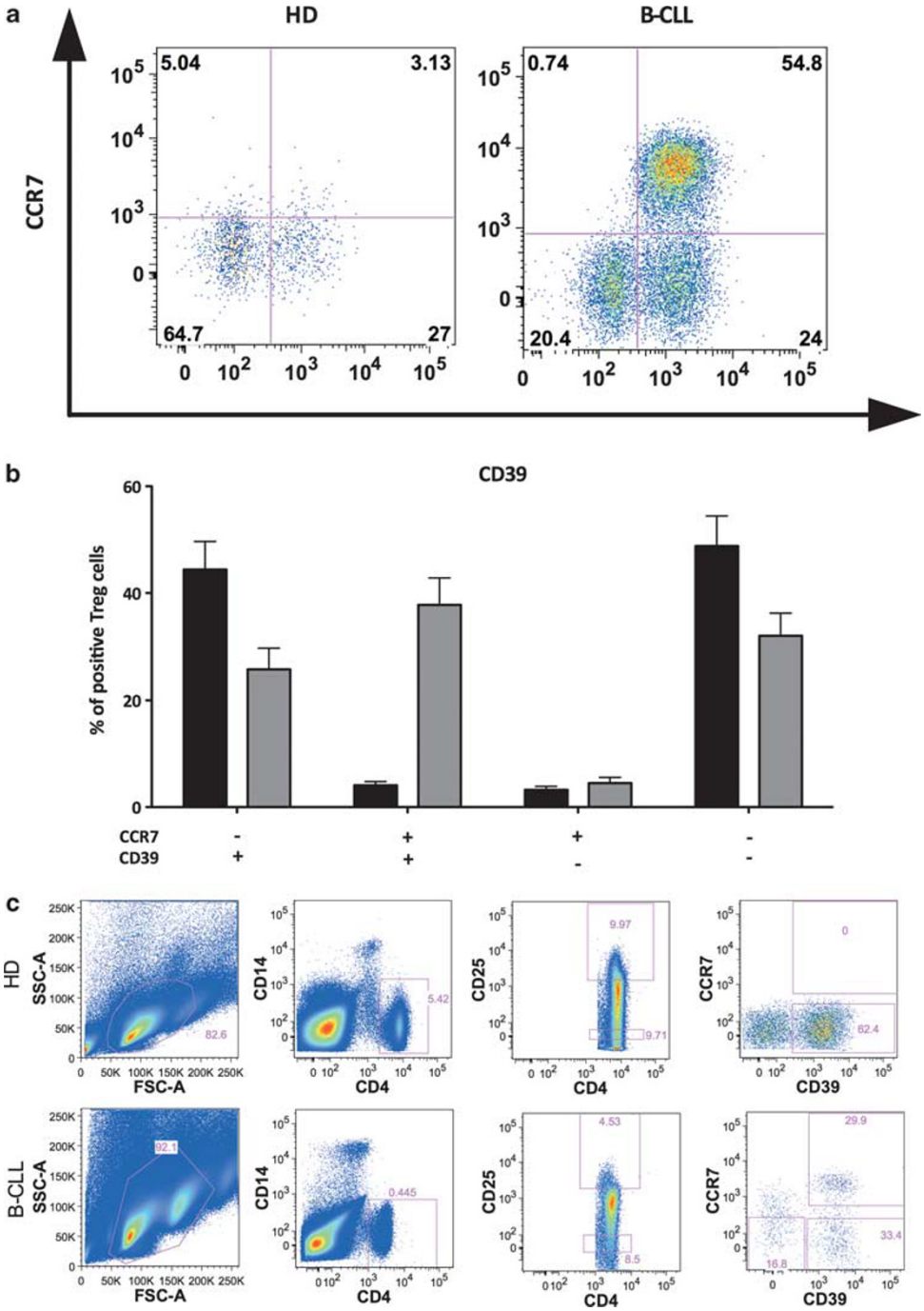


**Figure 3** Co-expression of CD39 and CCR7 in conventional T regulatory ($T_{regs}$) in healthy donors and chronic lymphocytic leukemia and autologous $T_{reg}$-mediated suppression of polyclonal T-cell responses from healthy donors and chronic lymphocytic leukemia. Conventional $T_{regs}$ were identified after staining as described earlier. Expression of the activation markers CD39 and CCR7 were measured. (**a**) Representative dot plot of CD39 and CCR7 co-expression pattern on conventional $T_{regs}$ in a healthy donor (left panel) and chronic lymphocytic leukemia (right panel). (**b**) Histograms of the mean percentages ( ± s.e.m.) of $T_{regs}$ expressing these markers in healthy donors (black) and chronic lymphocytic leukemia (gray). (**c**) $T_{regs}$ were isolated using flow cytometric sorting as described earlier. Expression of CD25 and CD4 were used to identify $T_{reg}$ cells in both healthy donors and in chronic lymphocytic leukemia. A representative dot plot of CD39 and CCR7 expression pattern in a healthy donor (upper panel) and chronic lymphocytic leukemia (lower panel). (**d**) Suppressive capacity of $T_{regs}$ toward responder cells ($T_{effec}$) was expressed as relative inhibition of the percentage of CSFE-low cells.

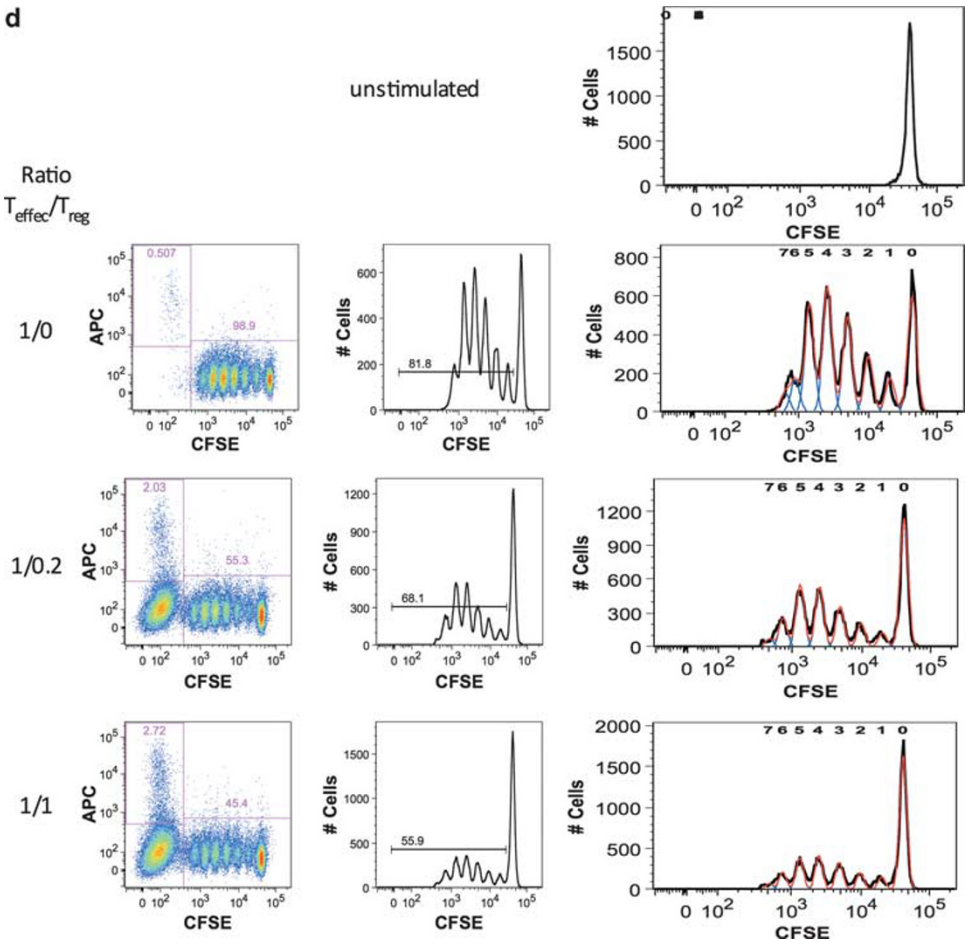


**Figure 3** Continued.

suppressed the proliferation of autologous responder cells in coculture in a dose-dependent manner, the mean relative inhibition for $T_{effec}/T_{reg}$ ratio 1:1 was $22 \pm 7.1\%$ for healthy donors. Among the chronic lymphocytic leukemia patients the mean suppression at a 1:1 $T_{resp}/T_{reg}$ ratio was $16.4 \pm 2\%$. The difference between the mean suppression in healthy donors and in chronic lymphocytic leukemia for $T_{effec}/T_{reg}$ ratio 1:1 was not significant ($P = 0.43$). In healthy donors, there are virtually no $CD39^+CCR7^+$ $T_{regs}$, thus, we were not able to sort this specific population for the proliferation assay experiment, and it was impossible to perform this control. In chronic lymphocytic leukemia, the relative inhibition of $CD39^+CCR7^+$ $T_{reg}$ was measured as performed for total $T_{reg}$, and was compared with the suppression obtained by the total $T_{reg}$ population in chronic lymphocytic leukemia. A trend to slightly less suppression was observed in this $T_{reg}$ sub-population compared with the total $T_{reg}$ population in chronic lymphocytic leukemia (1:1 mean suppression $= 12.5 \pm 1.2\%$, ($P = 0.59$)). Comparing the mean suppression of $CD39^+CCR7^+$ $T_{reg}$ with total $T_{reg}$ in healthy donors, showed a trend to a lower suppressive capacity, $12.5 \pm 1.2\%$, *vs* $22 \pm 7.1\%$, but did not reach significance ($P = 0.24$).

### PSM of $CD4^+$ and $CD8^+$ T-Cell Subsets in Healthy Donors and Chronic Lymphocytic Leukemia

Understanding the relationship of multiple markers to each other using bivariate plots and Boolean gating is difficult, particularly when attempting to evaluate 15-color immunophenotyping. To better understand these data, probability state models were constructed for $CD4^+$ and $CD8^+$ T cells in healthy donors and in chronic lymphocytic leukemia (Figure 1d). The use of these models emphasized the difference in the relative intensity of each marker in all populations from healthy donors and chronic lymphocytic leukemia, allowing an understanding of the behavior of each marker at through the progression to maturation. These models display intensity and frequency as a continuum for each marker studied to show the progression of $CD4^+$ or $CD8^+$ T cells from naïve to memory and ECs. The discrimination between memory and naïve population was performed according to the gating

**Table 3** Expression of activation markers among the naïve/memory $T_{regs}$ sub-populations

| | *HD* | *B-CLL* | P-*value* |
|---|---|---|---|
| *Naïve* | | | |
| CD38 | 2.3 ± 0.5 | 10.3 ± 3 | 0.002 |
| CD39 | 13.5 ± 2.5 | 81 ± 5.1 | <0.0001 |
| CD103 | 21.6 ± 3.2 | 14.6 ± 3.1 | <0.0001 |
| CD127 | 11.9 ± 2.5 | 31.1 ± 3.1 | <0.0001 |
| HLA-DR | 15.6 ± 1.6 | 71.9 ± 5.2 | <0.0001 |
| *Central memory* | | | |
| CD38 | 24.1 ± 2.7 | 20.4 ± 3.7 | 0.21 |
| CD39 | 42.5 ± 3.2 | 54.7 ± 6.4 | 0.1 |
| CD103 | 30.6 ± 3.8 | 6.4 ± 1.7 | <0.0001 |
| CD127 | 10.6 ± 1.7 | 43.6 ± 5.9 | <0.0001 |
| HLA-DR | 35.6 ± 2.6 | 24.1 ± 3.2 | 0.0022 |
| *Effector memory* | | | |
| CD38 | 3.2 ± 0.4 | 4.6 ± 0.8 | 0.2 |
| CD39 | 52.3 ± 3.5 | 46.7 ± 5 | 0.6 |
| CD103 | 21.05 ± 3.5 | 2.65 ± 0.7 | |
| CD127 | 17.9 ± 3 | 19.9 ± 2 | 0.16 |
| HLA-DR | 9.8 ± 0.9 | 2.7 ± 0.6 | <0.0001 |
| *Effector* | | | |
| CD38 | 4.3 ± 0.6 | 6.8 ± 1.4 | 0.06 |
| CD39 | 42.5 ± 3.1 | 54.7 ± 6.3 | 0.02 |
| CD103 | 5.1 ± 0.8 | 2.6 ± 0.5 | 0.02 |
| CD127 | 10.6 ± 1.7 | 43.6 ± 5.9 | 0.1 |
| HLA-DR | 17.9 ± 1.8 | 5.7 ± 1.8 | <0.0001 |

Abbreviation: Treg, T regulatory cell.

described in Materials and methods. Figure 1d shows a comparison of the Ag-dependent partitioning of events as determined by CD45RA, CD197, and CD27 marker downregulations in both $CD4^+$ T cells (left panels and $CD8^+$ T cells right panels) using data from a single donor. For $CD4^+$ T cells, the healthy donor's naïve, CM, EM, and EC fractions were 24.9%, 5.6%, 61%, and 8.8%, respectively. The chronic lymphocytic leukemia fractions were 13%, 10%, 67.3%, and 9.7%, respectively. For $CD8^+$ T cells, the healthy donor's naïve, CM, EM, and EC fractions were 37.6%, 3.7%, 53.6%, and 5.1%, respectively. The chronic lymphocytic leukemia fractions were 36.2%, 1.9%, 52.4%, and 10.6%, respectively. The chronic lymphocytic leukemia patient had decreased naïve and CM phases as well as increased EM and EC phases compared with the healthy donor patient.

### PSM of $T_{regs}$ Cells in Healthy Donors and Chronic Lymphocytic Leukemia

To simplify and better understand the complex relationship of marker expressions among $T_{reg}$ populations, PSMs were designed for $T_{reg}$ cells as described in a previous section (Figure 4a). An increase in the proportion of naïve $T_{reg}$ cells was observed in chronic lymphocytic leukemia patients compared with healthy donors. Activation markers such as CD39, CD38, and HLA-DR presented a higher relative intensity in chronic lymphocytic leukemia patients compared with healthy donors (Figures 4b–d). CD25 and CD127 presented a different pattern of expression in the EM $T_{reg}$ compartment (Figure 4e).

### FOXP3 Expression in $CD4^+CD25^-$ T Cells in Chronic Lymphocytic Leukemia Patients

During the evaluation of the numbers of $T_{reg}$ cells in healthy donors and in chronic lymphocytic leukemia patients, it was noted that among the $CD4^+$ T-cell population (Figure 1a) there was a cell population expressing FOXP3 but not CD25 ($CD4^+FOXP3^+CD25^-$). The percentage of these $CD25^-$ $FOXP3^+$ was significantly increased in chronic lymphocytic leukemia patients compared with healthy donors (4 ± 1% *vs* 1.45 ± 0.1%, $P = 0.0015$; Figure 5a). As for natural $T_{regs}$, the proportion of naïve, EC, CM and EM cells was determined for the $CD4^+FOXP3^+CD25^-$ cells in chronic lymphocytic leukemia (Figure 5b). In chronic lymphocytic leukemia patients, 29.9 ± 4.5% of $CD4^+FOXP3^+CD25^-$ cells were naïve, but the majority of these $CD4^+FOXP3^+CD25^-$ cells did not express CD45RA (70.1 ± 4.5%). Among $CD4^+FOXP3^+CD25^-$ cells, EM cells were predominant (57.8 ± 5.4%), followed by CM (9.3 ± 1.4%), and ECs (2.9 ± 0.6%).

A trend toward a higher expression of CD127 among the $CD4^+FOXP3^+CD25^-$ cells was observed in chronic lymphocytic leukemia patients compared with healthy donors (24.2 ± 3.4% in chronic lymphocytic leukemia *vs* 3 ± 0.4% in healthy donors ($P<0.001$)) and lower expressions of the activation marker HLA-DR was observed compared with healthy donors (52 ± 3.4% in healthy donors *vs* 32.6 ± 4.8% in chronic lymphocytic leukemia, $P<0.001$; Figure 5c). No significant differences were observed between healthy donors and chronic lymphocytic leukemia for the expression of CD103, CD39, and CD38 in $CD4^+FOXP3^+CD25^-$ cells.

### FOXP3 Expression in $CD8^+CD25^+$ T Cells in Chronic Lymphocytic Leukemia Patients

Within the $CD8^+$ T-cell population, a cell population expressing FOXP3 and CD25 ($FOXP3^+CD25^+$) was identified. The percentage of these $CD8^+$ $FOXP3^+CD25^+$ cells was significantly increased in chronic lymphocytic leukemia patients compared with healthy donors (11.2 ± 2.1% *vs* 0.4 ± 0.08%, respectively, $P<0.0001$; Figure 5d). The proportion of $CD8^+FOXP3^+CD25^+$ cells defined as naïve, EC, CM, and EM cells was determined for chronic lymphocytic leukemia, but not for healthy donors, as there were not sufficient numbers of cell with this phenotype in the latter for this task (Figure 5e). The majority of $CD8^+FOXP3^+CD25^+$cells in chronic

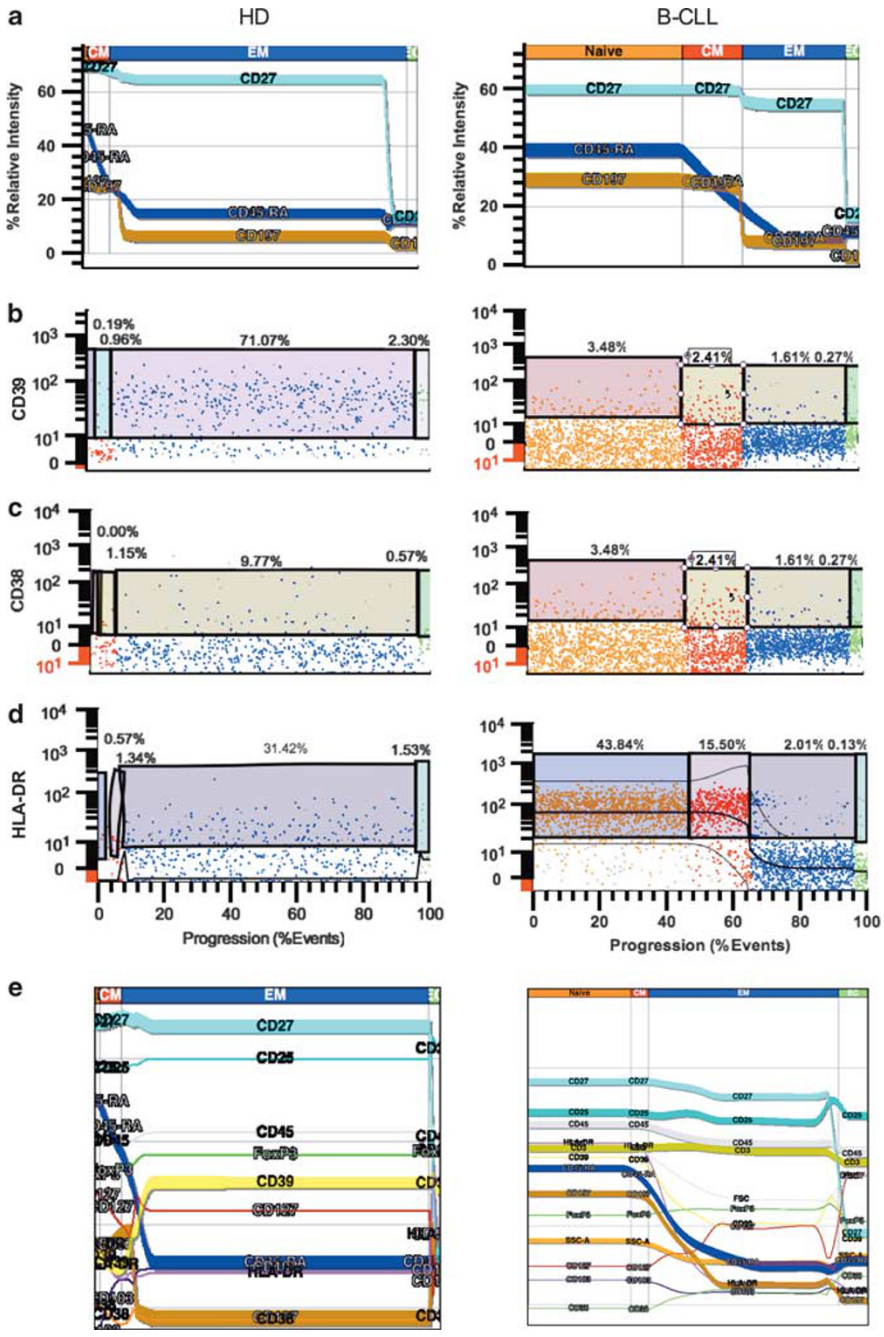


**Figure 4** Probability state modeling of T regulatory ($T_{reg}$) populations in healthy donors and chronic lymphocytic leukemia. Probability model of activation markers expression in conventional $T_{regs}$ in a healthy donor (left panel) and chronic lymphocytic leukemia (right panel). For these models, the data were gated on light scatter, viability, and expression of CD45, CD3, CD4, $CD25^{high}$, and FOXP3 as described earlier. The *y*-axis is relative fluorescence intensity, whereas the *x*-axis displays staged progression from naïve to memory and effector populations. The width of each stage (naïve, CM, EM, EC) indicates the percentage of cells falling into these categories. The width of the bands corresponds to the variability of the data as the cells progress. These graphs reveal the coordinate expression patterns of memory and naïve markers (**a**) as well as activation markers through the transitions (**b**, **c** and **d**). Panel **e** reveals the expression of all markers among the conventional $T_{reg}$ cells in a coordinated manner, with intensities of all markers revealed through the transitional steps.

lymphocytic leukemia were naïve, showing expression of $CD45RA^+$ ($76.4 \pm 4.1\%$). Approximately equal proportions of CM cells ($11.3 \pm 2.9\%$) and EM ($10.4 \pm 2.3\%$) were observed among $CD8^+FOXP3^+CD25^+$ cells. Effector cells represented $1.9 \pm 0.7\%$ of the $CD8^+FOXP3^+CD25^+$cells. Determination of naïve and memory sub-populations of $CD8^+FOXP3^+CD25^+$cells in healthy individuals was not possible due to the extremely low percentage of these cells found in healthy donors.

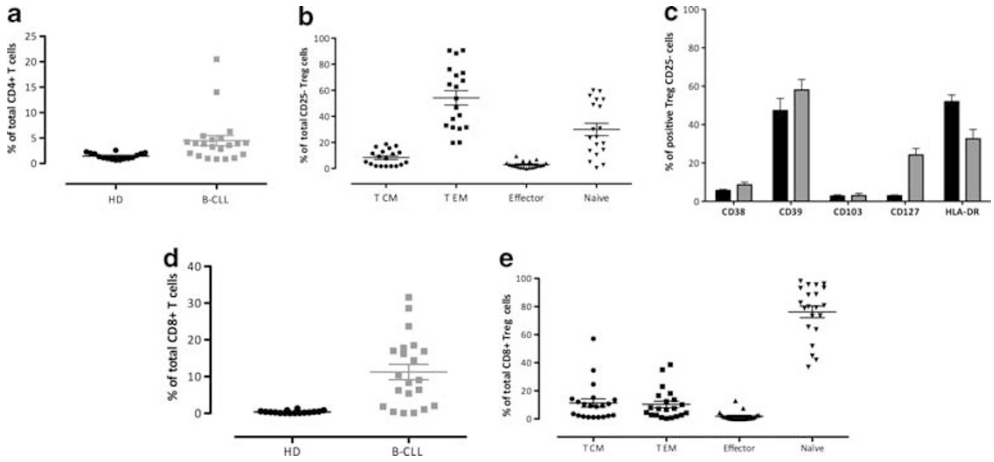


**Figure 5** $CD4^+CD25^-T_{regs}$ and CD8 $T_{regs}$, their memory *vs* naïve subtypes, and their activation status in healthy donors and chronic lymphocytic leukemia. Cells were stained with antibodies and analysis was performed to identify $CD25^-T_{regs}$ and CD8 $T_{regs}$ as described earlier. (**a**) The graphs display the percentages and the mean percentage values (± s.e.m.) of $CD25^-T_{regs}$ in healthy donors (black) and chronic lymphocytic leukemia (gray). (**b**) The graphs show the percentages and the mean percentage values (± s.e.m.) of central memory (CM) $CD25^-T_{regs}$ (identified as $CD27^+CCR7^+$ cells among $CD45RA^-CD25^-T_{regs}$), of effector memory (EM) $CD25^-T_{regs}$ (identified as $CD27^+CCR7^-$ cells among $CD45RA^-CD25^-T_{regs}$), of effector $CD25^-T_{regs}$ (identified as $CD27^-CCR7^-$ cells among $CD45RA^-CD25^-T_{regs}$), and naïve $CD25^-T_{regs}$ (identified as cells among $CD25^-T_{regs}$). (**c**) The graphs display the mean percentage values (± s.e.m.) of $CD25^-T_{regs}$ expressing activation markers CD38, CD39, CD103, CD127, and HLA-DR in healthy donors (black) and chronic lymphocytic leukemia (gray). (**d**) The graphs represent the percentages and the lines indicate the mean percentage value (± s.e.m.) of CD8 $T_{regs}$ in healthy donors (black) and chronic lymphocytic leukemia (gray). (**e**) The graphs show the percentages and the lines indicate the mean percentage value (± s.e.m.) of CM CD8 $T_{regs}$ (identified as $CD27^+CCR7^+$ cells among $CD45RA^-$CD8 $T_{regs}$), of effector memory (EM) CD8 $T_{regs}$ (identified as $CD27^+CCR7^-$ cells among $CD45RA^-$CD8 $T_{regs}$), of effector CD8 $T_{regs}$ (identified as $CD27^-CCR7^-$ cells among $CD45RA^-$CD8 $T_{regs}$), and naïve CD8 $T_{regs}$ (identified as $CD45RA^+$ cells among CD8 $T_{regs}$).

### Correlation of Immunophenotypes with Prognostic Markers

The frequencies of $T_{regs}$ and of the various $T_{reg}$ subsets were analyzed for their correlation with known prognostic markers. Previous studies have reported that a combination of CD38 and ZAP-70 is the best predictor of disease progression.[33–35] Using the Spearman's correlation, we found that both $CD45RA^+$ $T_{reg}$ and CD8 $T_{reg}$ were significantly inversely correlated with combined CD38 and ZAP-70 expression (Spearman's $r=-0.55$ ($P=0.02$) and Spearman's $r=-0.58$ ($P=0.01$), respectively). Using the Mann–Whitney test, we compared the mean of these two populations in two groups $CD38^-/ZAP70^-$ and $CD38^+/ZAP70^+$, and it was found that the frequency of $CD45RA^+$ $T_{regs}$ and $CD8^+$ $T_{regs}$ was significantly different. $CD45RA^+$ $T_{reg}$ cells were lower in $CD38^+/ZAP70^+$ group; $41\pm6.9\%$ *vs* $60\pm5.3\%$ in $CD38^-/ZAP70^-$ ($P=0.02$). CD8 $T_{reg}$ cells were also lower in $CD38^+/ZAP70^+$ group; $6.7\pm2.1\%$ *vs* $17.7\pm3\%$ in $CD38^-/ZAP70^-$ ($P=0.02$). We did not observe any other significant correlation between the various $T_{reg}$ phenotypes and the combination of predictors of disease progression.

## Discussion

T-regulatory cells can be identified in a number of different ways. Bright expression of CD25 alone on CD4 T cells or in concert with low CD127 expression or expression of FOXP3 are the most common methods of identification of $T_{regs}$. Among the reports of $T_{regs}$ in chronic lymphocytic leukemia, only Giannopoulos *et al*[10] used FOXP3 as a component of $T_{reg}$ identification, yet all found an increase in the frequency of these cells in chronic lymphocytic leukemia. In concordance with these studies we found an increase in the frequency of $T_{regs}$ in the peripheral circulation compared with age-matched healthy donors. The literature is divided on whether the frequency of $T_{regs}$ in chronic lymphocytic leukemia correlates with prognostic markers such as CD38, ZAP-70, or IgVH mutational status, and it remains unclear why these discrepancies occur. Several studies did find a correlation between the frequency of $T_{regs}$ and clinical stage,[6,10] but we found that the frequency of total $CD4^+$ $T_{regs}$ did not significantly correlate with prognostic markers. We found instead that specific sub-populations of $T_{regs}$, $CD4^+CD45RA^+$ $T_{regs}$, and $CD8^+$ $T_{regs}$ did show a correlation. These findings strongly suggest that elucidating the complex immunophenotypes of $T_{regs}$ found in chronic lymphocytic leukemia is necessary to understand their role in this disease progression.

We observed a significant increase of $CD4^-CD8^-$ T cells in patients with chronic lymphocytic leukemia, these cells could be immature T cells, and could reflect the change on T-cell function induced by the presence of chronic lymphocytic leukemia cells. In addition to immature T cells, these double-negative T cells could be iNKT cells, or γδ T cells.

Further experiments would be needed to determinate further the nature of these cells.

The expression of FOXP3 as a specific marker of $T_{regs}$ cells is controversial regarding whether it is sufficient for a suppressor phenotype.[36] Recent data supports the concept that transient expression of FOXP3 does not confer suppressive activity but permanent expression does.[12] The current data demonstrate that $CD4^+CD25^-FOXP3^+$ and $CD8^+CD25^{high}FOXP3^+$ populations were significantly increased in chronic lymphocytic leukemia patients compared with healthy donors. Unfortunately there is no way to know whether this FOXP3 expression is transient or permanent. Furthermore, as these cells could only be identified through the use of intracellular staining for FOXP3, they could not be sorted as viable cells and thus there is no way of definitively establishing their suppressive function. Both $CD4^+CD25^-FOXP3^+$ as well as $CD8^+CD25^+FOXP3^+$ $T_{regs}$ have previously been described[37–39] and it is within reason to think these subsets may have suppressive functions. If these populations are indeed suppressive, this suggests that previous studies have underestimated the frequency of $T_{regs}$ in chronic lymphocytic leukemia as those studies only identified $CD4^+CD25^+$ cells as $T_{regs}$.

Within the $CD4^+CD25^{high}FOXP3^+$ $T_{regs}$ there exists a greater complexity of phenotypes than reported to date. Until recently, $CD4^+CD25^+T_{reg}$ cells have been described using limited markers as belonging to the memory T-cell compartment.[13–16] Valmori *et al*[40] identified a $T_{reg}$-cell population with naïve phenotype ($CCR7^+CD45RA^+$), which they termed natural naïve $T_{reg}$ cells. Several observations have been made by other groups examining the naïve ($CD45RA^+$) *vs* memory ($CD45RO^+$) phenotype of $T_{regs}$ population. Among these observations are the findings of: (1) an increase of memory $T_{regs}$ in older HIV-infected patients,[41] (2) an increase of naïve $T_{regs}$ in younger healthy donors,[41] and (3) that the frequency, phenotypic characteristic, and antiproliferative function were comparable in the young (age $<40$ years) and the elderly (age $>65$ years).[42,43] The current data demonstrate that $T_{regs}$ can readily be classified as naïve, EC, CM, and EM subsets using CD27, CCR7, and CD45RA. The $NnT_{reg}$ population, identified in the current study as viable $CD45^+CD3^+CD8^-CD4^+CD25^+FOXP3^{high}CD45RA^+CCR7^+CD27^+$, is increased in the chronic lymphocytic leukemia patients compared with controls. Statistically significant differences between chronic lymphocytic leukemia patients and healthy donors were observed in EM $T_{regs}$ as well as in effector $T_{regs}$ using these classifications. The data suggest that simple identification of $T_{regs}$ as naïve or adaptive may be too superficial to draw meaningful conclusions regarding their function, as these cells lend themselves to further phenotypic compartmentalization as performed for $CD4^+$ T cells as a whole. By using these complex classifications, significant differences are observed in the $T_{reg}$ populations between chronic lymphocytic leukemia patients and healthy donors. The current data show in chronic lymphocytic leukemia patients averaging over 65 years of age, naïve $T_{regs}$ remain a predominant population, whereas in healthy donors, we showed a paucity of naïve $T_{regs}$, consistent with previous reports. Booth *et al*[44] have demonstrated that human $T_{regs}$ expressing either CD45RA or CD45RO have identical suppressive capacity in healthy individuals, although the former are less proliferative. Interestingly, their data also show that CD45RA $T_{regs}$ express CXCR4 and preferentially accumulate in the bone marrow.

Within the $T_{reg}$ population, the chronic lymphocytic leukemia patients had patterns of marker expression, other than CD45RA, differing significantly from those in healthy donors, such as increased co-expression of CD39 and CCR7. CD39 has been closely associated with the suppressive activity of $T_{regs}$.[45] Thus, dual-positive $CCR7^+CD39^+$ $T_{regs}$ were speculated to be a highly suppressive phenotype. In five cases of chronic lymphocytic leukemia, the suppressive capability of the sorted $T_{regs}$ $CD39^+CCR7^+$ was less than the total $T_{regs}$ from the same donor but this did not reach statistical significance. It was not possible to sort sufficient $CD39^+CCR7^+$ $T_{regs}$ from healthy donors, as virtually none existed. However, compared with total $T_{regs}$ from healthy individuals, the $CD39^+CCR7^+$ $T_{regs}$ unexpectedly showed a slightly reduced suppressive capability. These $CD39^+$ $T_{regs}$ might be highly activated but not necessarily highly suppressive. Another observation was an increased expression of CD127 in the $T_{regs}$ from chronic lymphocytic leukemia. Studies have shown that CD127 has an important role in the proliferation and differentiation of mature T cells, and *in vitro* experiments show that the expression of CD127 is downregulated following T-cell activation.[46,47] Earlier studies showed that CD127 expression inversely correlates with the suppressive function of $T_{regs}$.[48] The increase of CD127 could be linked to the naïve phenotype of our chronic lymphocytic leukemia $T_{regs}$, as low expression of CD127 is a marker for natural $T_{regs}$ that have a memory phenotype,[7] and the chronic lymphocytic leukemia $T_{regs}$ have a naïve phenotype. This finding would suggest that care should be taken when using low CD127 expression to define $T_{regs}$ in these patients, and possibly in patients with other forms of cancer. Taken as a whole, our data indicate that it is possible to delineate many putative subtypes of $T_{regs}$ cells using widely studied markers.[49]

In the context of chronic lymphocytic leukemia, as well as other neoplastic diseases, ascribing the regulation of immune responses by $T_{regs}$ might be a far more complex process than simply measuring $CD4^+CD25^+FOXP3^+$ cells. Much work remains to validate such speculation by sorting the populations of $T_{regs}$ identified by the 15-color immunophenotyping and elucidating the function of each. It is hoped

that the high-dimensional immunophenotyping of $T_{regs}$ demonstrating numerous distinct sub-populations presented here will serve as an impetus for such studies.

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## Disclosure/conflict of interest

The authors declare no conflict of interest.

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