Chapter 3

Enhanced formation and survival of CD4^+CD25^hiFoxp3^+ T cells in CLL

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Abstract

Recently it has been described that chronic lymphocytic leukemia (CLL) patients have increased numbers of regulatory T cells (Treg). In the present study, we analysed the mechanism behind Treg expansion in CLL. Neither analysis of the T cell receptor (TCR) repertoire nor CD45 isoform expression of Treg from CLL patients provided evidence for chronic (tumor) antigenic stimulation as a possible cause for Treg expansion in CLL. We found evidence however for increased formation of Treg via CD70 costimulation, since we observed that CD40 ligand activated CLL cells (which might be considered a model of lymph node CLL cells) strongly induced CD70-dependent formation of Treg.

RT-MLPA expression analysis of 34 apoptosis-regulating genes showed that in comparison to other CD4+ T cells, Treg from both healthy individuals and CLL patients had a high expression of pro-apoptotic Noxa and a low expression of anti-apoptotic Bcl-2. Strikingly, Bcl-2 levels of Treg in CLL patients were significantly higher than in healthy individuals. Finally, the different apoptotic profile resulted in differences at the functional level, since Treg from CLL patients were more resistant to drug-induced apoptosis than Treg from healthy individuals.

In conclusion, Treg in CLL may accumulate both by increased formation, facilitated by CD27-CD70 interaction in the lymph node proliferation centres, and decreased sensitivity to apoptosis due to a shifted Noxa-Bcl-2 balance.
Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the slow accumulation of mature CD5+CD19+ B cells. Notably, CLL patients frequently also have increased numbers of circulating CD4+ and CD8+ T cells. At present, there are no data supporting CLL-specificity of these expanded T cell populations. In contrast, we have shown that CLL patients can have increased numbers of CMV-specific CD8+ memory effector cells.

Recently it has been described that CLL patients have increased numbers of CD4+CD25bright regulatory T cells (Treg), with highest Treg frequencies in progressing patients with extended disease. Importantly, Treg cells from CLL patients show inhibitory function similar to healthy controls. Tregs are thought to play an important role in immune evasion by malignancies. High Treg numbers in patients with malignancies are often associated with poor prognosis. Since high Treg numbers in CLL might negatively affect the course of disease, it will be important to elucidate the mechanism of this Treg expansion. Most naturally occurring Foxp3+ Tregs in adults express markers of primed T cells, such as CD45R0. The naïve marker CD45RA, which is expressed by a proportion of naturally occurring Foxp3+ Tregs, decreases with age. In contrast to naturally occurring Treg which are generated in the thymus, the majority of Treg in healthy adults are generated in the periphery. These so-called adaptive or induced Treg are likely to be continuously produced from the memory CD4+ T cell pool, since most Treg possess a memory phenotype (CD45R0+) and the T cell receptor repertoire of Treg shows a high homology to that of the CD4+ memory T cell pool. However, the turnover of Treg appears to be much faster than that of memory T cells and since in healthy individuals there is no steady increase in Treg numbers the increased proliferation rate apparently is counterbalanced by regulated apoptosis of Treg. Thus, the increase of Treg in CLL might be due either to increased formation (either by increased proliferation or by differentiation of non-Tregs into Tregs), decreased apoptosis or both. Another possibility of an increase in Tregs in the peripheral blood of CLL patients is redistribution of Tregs; by distorting lymph node architecture tumor cells could drive Tregs out of the secondary lymphoid organs into the blood.

In the present study we have examined potential mechanisms of increased Treg numbers in CLL. Possible expansion by chronic antigenic stimulation was evaluated by analysis of the T cell receptor repertoire and the expression of differentiation markers on Treg. Furthermore, we investigated whether CLL cells themselves play a stimulatory role in the formation of Treg. Finally, alterations in apoptosis of Treg were studied by expression profiling of 34 apoptosis regulating genes as well as by assessment of Treg sensitivity to cytotoxic drugs.
Methods

Cells from CLL patients and healthy individuals

Peripheral blood was drawn from CLL patients (diagnosed according to the NCI-WG guidelines) as well as from healthy volunteers (blood bank donors). PBMC were isolated and either used immediately or stored in liquid nitrogen. During all in vitro experiments, cells were maintained in Iscove’s modified Dulbecco medium (IMDM: Gibco Life technology, Paisley, USA) supplemented with 10% heat-inactivated fetal calf serum, penicillin, gentamycin and β-mercaptoethanol. All PBMC samples from CLL patients contained at least 90% CD5+/CD19+ cells as assessed via flow cytometry. The age range of CLL patients was 28-86 years (female: male ratio 1:1.6) and of healthy donors 29-61 years. (F:M ratio 1:0.8). The studies were approved by the Ethical Review Board of the Institute and from all participants written informed consent was obtained. Patient characteristics are listed in table 1.

Table 1. Patient characteristics

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Patient characteristics including gender, age, Rai stage, mutation of IgVH genes and prior therapy. (F= female, M = male, Mut = mutated IgVH genes, Unmut = unmutated IgVH genes, ND = not determined, C= cyclophosphamide, CA= chlorambucil, F= fludarabine, R= rituximab, no Tx = no therapy). Patients indicated with an asterisk (*) showed highly progressive disease.
Flow cytometry
PBMC were stained using antibodies against CD4, CD8, CD5, CD19, CD25, CD45RA or CD45R0 and CD127 (all Becton Dickinson, San Jose, CA), CD70 FITC (clone CLB-2F2) or with antibodies against CD95 (IQ products, Groningen, the Netherlands). For intracellular staining, cells were fixed and permeabilised (eBioscience, San Diego, CA) and subsequently stained for Bcl-2 (Dako, Glostrup, Denmark), Ki-67 (BD Pharmingen, San Jose, CA) and Foxp3 (eBioscience, San Diego, CA). An isotype-matched control antibody for Bcl-2 and CD95 we used mouse IgG1 FITC, clone X40 (BD Pharmingen, San Jose, CA) and for Ki-67 we used FITC-conjugated mouse IgG1, κ isotype control, clone MOPC-21 (BD Pharmingen, San Jose, CA). Antibody stained cell samples were analysed by flow cytometry with the CellQuest program on a fluorescence-activated cell sorting (FACS) Calibur (Beckton Dickinson).

Isolation of T cell populations
Thawed PBMC from either healthy individuals or CLL patients were stained with antibodies against CD4, CD25 and CD127 (all Becton Dickinson, San Jose, CA). Subsequently, Treg (CD4+/CD25bright/CD127low) and non-Treg CD4+ T cells (CD4+/CD25-/CD127+) were obtained by cell sorting (FACS Aria, Becton Dickinson, San Jose, CA). The isolated cells were immediately lysed to prepare RNA or perform protein isolation. Treg enriched populations contained approximately 80% CD4+/Foxp3+ cells as assessed by flow cytometry.

Analysis of Vβ repertoire
RNA isolated from sorted T cells was subjected to template switch-anchored reverse transcriptase–polymerase chain reaction (RT-PCR) by using Super Smart PCR cDNA Synthesis Kit (BD Biosciences Clontech, Palo Alto, CA). Vβ PCR was performed on amplicons as described previously.[12] For the spectratyping, samples were mixed with Genescan-500 ROX size standards and run on an ABI 3100 capillary sequencer (Applied Biosystems, Warrington, United Kingdom) in Genescan mode.

In vitro CD40 ligand stimulation of CLL cells
PBMC from CLL patients (> 90% CD5+CD19+ cells) were stimulated with CD40 ligand (CD40L) transfected NIH3T3 (3T40L) cells as described previously.[13] Briefly, CLL cells were added to 6-well plates coated with gamma irradiated (30 Gy) CD40L transfected NIH3T3 cells. Non-transfected 3T3 cells were used as negative controls. After 2 days, the CLL cells were gently removed from the fibroblast layer and used in further experiments.

Cell stimulation cultures (CSC)
CSC were performed with 3T3- or 3T40L-stimulated CLL cells (APC) and PBMC of a healthy individual or (autologous) CLL patient (responders) in a 1:1 ratio (2 x 10^5 stimulators: 2 x 10^5 responders). Cells were cultured in 96-wells plates (Costar, Corning Inc., NY, USA) in the presence of soluble CD3 mAb (clone CLB-T3.4/E)[14] and in the presence or absence...
of a blocking CD70 mAb (clone CLB-2F2)\textsuperscript{15}. After 4 days cells were harvested and Foxp3 expression was analyzed by flow cytometry as described above.

**Reverse transcription–multiplex ligation-dependent probe amplification assay**

Reverse transcription–multiplex ligation-dependent probe amplification assay (RT-MLPA) procedure was performed as described previously\textsuperscript{16}. Briefly, 100 ng total RNA as obtained from sorted T cell populations was reverse transcribed using a gene-specific probe mix. The resulting cDNA was annealed overnight at 60°C to the MLPA probes. Annealed oligonucleotides were covalently linked by Ligase-65 (MRC, Amsterdam, The Netherlands) at 54°C. Ligation products were amplified by polymerase chain reaction (PCR; 33 cycles, 30 seconds at 95°C, 30 seconds at 60°C, and 1 minute at 72°C) using one unlabeled and one 6-carboxy-fluorescein–labelled primer (10 pM). PCR products were size separated on an ABI 3100 capillary sequencer in the presence of 1 pM ROX 500 size standard (Applied Biosystems, Warrington, United Kingdom). Results were analyzed using the programs Genescan analysis and Genotyper (Applied Biosystems). Category tables containing the area for each assigned peak (scored in arbitrary units) were compiled in Genotyper and exported for further analysis with Excel spreadsheet software (Microsoft, Redman, WA). Data were normalized by setting the sum of all signals at 100% and expressing individual peaks relative to the 100% value. The thus obtained expression levels of all tested genes in Treg populations (see isolation of T cell populations) were then compared to the levels found in the non-Treg CD4\textsuperscript{+} T cell population and reflected as relative expression (gene expression in CD4 set as 1).

**Quantitative PCR analysis of Noxa expression**

20 ng of RNA extracted from sorted cell populations (see analysis of V\textsubscript{\beta} repertoire) was used to synthetize cDNA with superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). From these cDNA pools, specific targets were amplified by PCR performed with Lightcycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Indianapolis, IN), using the sense and antisense Noxa primers 5’-GGAGATGCTGGGAAGAAGG-3’ and 5’-TCAGGTTTGGATGGAGAG-3’ and the 18S primers 5’-GGACAACAGCTCCGTGAAGA-3’ and 5’-CAGAAGTGACGGAGCCCTCTA-3’ respectively. The results were normalized to 18S. Thus obtained values for Treg were set as 1 and compared to values of non-Treg (relative expression).

**Western blotting**

Western blotting was performed as described previously\textsuperscript{17}. Protein samples were separated by 13% sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Western blotting. Blots were probed with the following antibodies or antisera: polyclonal Mcl-1 (BD Pharmingen, San Jose, CA), monoclonal anti-Noxa (Imgenex, San Diego, CA), anti-Bcl-X\textsubscript{L} (Transduction Laboratories, Lexington, KY), rabbit-anti-Bcl-2 (Alexis Biochemicals, San Diego, CA) or antiserum to \(\beta\)-actin (Santa Cruz Biotechnology, Santa Cruz, CA).
Drug sensitivity assays
PBMC of both CLL patients and healthy individuals were incubated with various concentrations of fludarabine (Sigma-Aldrich, St Louis, MO), Roscovitine (Sigma Aldrich, St Louis, MO) or agonistic Fas antibody CH11 (Beckman Coulter, Fullerton, CA). After 24 hours, cells were fixed and permeabilised and stained for CD3, CD4, CD25 (all Beckton Dickinson) and Foxp3 (eBioscience) to determine the percentage of regulatory T cells (Treg) out of the total CD4 population. The obtained values were then normalized by calculating the percentage of Treg remaining after drug stimulation compared to non-stimulated as follows: \( \frac{\text{Foxp3}^+/(\text{CD4}^+)}{\text{drug stimulated}}/\frac{\text{Foxp3}^+/(\text{CD4}^+)}{\text{non-stimulated}} \times 100\% \).

Alternatively, drug-treated PBMC samples were fixed and analyzed for the presence of fragmented DNA (permeabilization buffer containing 0.1mM EDTA, 10\( \mu \)g ml\(^{-1} \) propidium iodide and 50\( \mu \)g ml\(^{-1} \) RNase-I) or cleaved caspase-3 (BD Pharmingen, San Jose, CA) within the Foxp3 positive and negative population.

Statistics
The two-tailed Mann-Whitney U test was used to analyze differences between 2 groups. Alternatively, the Wilcoxon matched paired test was used to analyze differences between paired samples. P-values < 0.05 were considered statistically significant.

Results
CLL patients have increased numbers of regulatory T cells in peripheral blood
In agreement with our previous studies in CLL patients we found increased numbers of CMV-specific CD8\(^+\)/CD45RA\(^-\)/CD27\(^-\) cells\(^3\) but no increase in the CMV-associated CD4\(^+\)/CD27/CD28 T cell population\(^18\) (data not shown). In addition, we observed an increase in numbers of CD4\(^+\)/CD25\(^{bright}\)/CD127\(^{low}\) T cells (figure 1A). This phenotype has been associated with Foxp3 expression and regulatory function\(^19,20\). Indeed, counterstaining with Foxp3 antibody showed predominantly Foxp3 positive T cells within the CD4\(^+\)/CD25\(^{bright}\)/CD127\(^{low}\) population (figure 1B), thereby confirming recent findings that CLL patients have increased numbers of regulatory T cells\(^4\) (Treg). In line with previous studies\(^4\) we observed higher Treg frequencies (defined as CD4\(^+\)/FoxP3\(^+\) cells) in patients with extended disease (Supplemental Figure S1).

No evidence for predominant antigen involved in Treg formation
A possible mechanism behind Treg formation and/or maintenance is (chronic) antigenic stimulation. Recently it has been demonstrated that upon antigenic stimulation, a limited number of Treg clones arises with the same T cell receptor (TCR) as the antigen-specific T cells from the effector cell pool\(^9\). Thus, an antigenic “fingerprint” is present within both the
effector T cell pool and the Treg population. To see whether a predominant antigen may be involved in the formation or maintenance of the Treg population in CLL patients, we screened the TCR repertoire recovered from Treg from healthy individuals (n=2) and compared this repertoire to that of non-Treg CD4+ T cells (combination of naive and memory CD4 cells) from the same individuals. We observed that the complete range of Vβ genes was used in Treg and non-Treg from both healthy individuals and CLL patients (figure 2A). We randomly chose 3 Vβ families for fragment length analysis (spectratyping). Also here, the 3 randomly chosen Vβ family PCR products showed similar peak patterns for both non-Treg and Treg cells (figure 2B) in all individuals, making the involvement of a predominant antigen in Treg formation in CLL patients less likely. Only within the Vβ11 family, a discrepant peak was observed in Treg compared to the non-Treg CD4+ T cell population, but this peak was present in Treg from both CLL patients and healthy individuals.

Next, we examined the “antigen experience” of Treg by determining the surface expression of CD45R0. In line with previous studies, we found that the vast majority of the Treg in these adult individuals have an antigen-experienced phenotype when compared to non-Treg T cells, as characterized by surface expression of CD45R0 (figure 2C). We observed no difference in the percentage of CD45R0 positive Treg between healthy individuals and CLL patients. The percentage of CD45R0+ cells in the non-Treg population was statistically different between healthy individuals and CLL patients (Figure 2C). This difference could neither be explained by age, nor by CMV status (data not shown).
CD40 ligand-stimulated CLL cells induce Foxp3 expression in CD4+ T cells in a CD70 dependent manner

Recently, it has been shown that CD70+ non-Hodgkin’s lymphoma (NHL) B cells can induce Treg via CD70 costimulation\(^{21}\). In contrast to peripheral blood CLL cells, CD40 ligand (CD40L) stimulated CLL cells (which resemble CLL cells from a lymph node environment\(^{22}\)) have high CD70 surface expression\(^{13,23}\). Therefore, we hypothesized that in a lymph node environment CLL cells might facilitate the formation of Treg. To test this, we performed cell stimulation cultures (CSC) using CLL cells that were pre-stimulated with 3T3 or CD40L transduced 3T3 cells (3T40L; see methods) as APC and PBMC from a healthy individual or autologous PBMC from CLL patients as responder cells. All CSC were performed in the presence of mitogenic CD3 mAb (see methods). After 4 days, cells were harvested and analyzed by flow cytometry. After CD40L-stimulation of CLL cells, both the percentage of CD5+CD19+CD70+ cells and CD70 mean fluorescence intensity (MFI) strongly increased (figure 3A). Strikingly, we observed that CD40L-stimulated CLL cells significantly augmented Foxp3 expression in CD4+ T cells of a healthy individual (* p= 0.019). Moreover, this augmentation could be blocked by anti-CD70-antibodies (2F2; figure 3B). To test if this effect was also present in an autologous setting, CD40L-stimulated CLL cells were used to stimulate autologous T cells. Also here, CD40L-stimulated CLL cells augmented Foxp3 expression in autologous CD4+ T cells (n=4), which could again be blocked by anti-CD70-antibodies (figure 3C). When paired CSC’s were analyzed, we showed that Foxp3 induction was CD70 dependent; when cultured in the presence of CD70 blocking antibodies, Foxp3 induction in CD4+ T cells of both healthy individuals and CLL patients was significantly inhibited (p=0.0156; figure 3 D). As a control we used anti-CD80-antibodies, which showed no significant inhibition of Foxp3 induction (data not shown).

High expression of Noxa and low Bcl-2 characterize a pro-apoptotic profile of Treg

Since Treg have been described to be highly susceptible to apoptosis\(^{11}\), we investigated whether this might be related to the expression of pro- or anti-apoptotic molecules. To establish the ‘apoptotic profile’ of Treg, both non-Treg CD4+ T cells and Treg from 3 healthy individuals were sorted based on IL2R and IL7R expression\(^{20}\). RNA was extracted from these T cell populations and used as input for RT-MLPA expression analysis to evaluate the expression levels of 34 apoptosis-regulating genes. We found that overall expression profiles in Treg were very similar to those in non-Treg CD4+ T cells (n=3; figure 4A). However, 2 genes had a markedly different expression in Treg. First, the levels of the pro-apoptotic BH3-only molecule Noxa were considerably increased in Treg as compared to non-Treg CD4+ T cells (2.89 fold increase; p=0.02). Secondly, in line with previous studies\(^{9}\) we found that Bcl-2 expression was significantly decreased in Treg as compared to non-Treg CD4+ T cells (3.02 fold decrease; p=0.01).
High mRNA levels of Noxa in Tregs were confirmed by quantitative PCR analysis (figure 4B). Protein analysis subsequently confirmed the elevated expression levels of Noxa and low expression of Bcl-2 in Treg (figure 4C). Thus, Treg seem to have a unique apoptotic profile which suggests enhanced susceptibility to apoptosis induction. Indeed, spontaneous cell death in purified Treg was higher than in non-Treg CD4⁺ T cells (figure 4D; left dot plots, non-treated). Moreover, when sorted Treg cells were treated overnight with a moderate concentration of Roscovitine (a drug that preferably induces apoptosis in cells with high Noxa levels24), they were much more sensitive to cell death than non-Treg CD4⁺ T cells (figure 4D; right dot plots, Roscovitine). Moreover, under these conditions, higher percentages of cleaved caspase 3 and DNA fragmentation (both classical hallmarks of apoptosis) were found in Tregs when compared to non-Treg CD4⁺ T cells (Figure 4E).

Treg from CLL patients are relatively protected against apoptosis

Since Treg have a highly apoptosis-prone gene expression profile, the increased number of Treg in CLL might be caused by small alterations in expression levels of apoptosis-regulating genes. Figure 5A shows the expression levels of 34 apoptosis-regulating genes of both Treg and non-Treg CD4⁺ T cells of 2 CLL patients. Similar to healthy controls Tregs from CLL patients have higher Noxa levels compared to non-Treg CD4⁺ T cells (figure 5A and B). Comparing the expression levels in Figure 4A (healthy controls) with levels in Figure 5A (CLL patients), we further analyzed 2 apoptosis regulating genes that showed differences between healthy controls and CLL patients (Bcl-2 and IAP1). Comparing expression levels of these genes in Treg populations to levels in non-Treg populations (with non-Treg CD4⁺ set as 1), we found that Treg from CLL patients express higher levels of Bcl-2 than Treg from healthy individuals (figure 5B). This was confirmed at the protein level by intracellular staining (figure 5C). This supports the notion that Treg from CLL patients might be relatively protected against apoptosis, since the elevated expression of Bcl-2 observed in Treg from CLL patients might serve to counterbalance the high expression of Noxa (figure 4A and B, figure 5B). The mechanism behind high Bcl-2 levels in Treg from CLL patients remains to be...
Figure 3 Cell Stimulation Cultures (CSC) and Foxp3 induction.

CSC were performed with 3T3 or 3T40L stimulated CLL cells (APC) and PBMC of a healthy individual (HD) or autologous PBMC from CLL patients (responders) in a 1:1 ratio. Cells were cultured in the presence of soluble CD3 mAb and in the presence or absence of a blocking anti-CD70 mAb (2F2). Foxp3 expression was analyzed after 4 days.

A. CD70 expression on CD5+/CD19+ cells before (t=0) and after 2 days co-culture with 3T3 or 3T40L (n=3). Left: percentage CD5+/CD19+/CD70+ cells ± SEM. Right: CD70 expression of CD5+/CD19+/CD70+ cells. Data are presented as mean fluorescence intensity (MFI) ± SEM. Top right: CD70 expression; overlay of 3T3 stimulated CLL cells (light grey line) and 3T40L stimulated CLL cells (black line). Gated on CD5+/CD19+ cells.

B. CSC with 3T3 stimulated CLL (3T3-CLL) or 3T40L stimulated CLL cells (3T40L-CLL) as APC and PBMC of HD as responders in presence (grey bars) or absence (white bars) of 2F2 (n=3). Data are presented as percentage CD4+/Foxp3+ cells (mean ± SEM). (* p= 0.019)

C. CSC of 3T3 stimulated CLL (3T3-CLL) or 3T40L stimulated CLL cells (3T40L-CLL) and autologous PBMC of CLL patient in presence (grey bars) or absence (white bars) of 2F2 (n=4). Data are presented as percentage CD4+/Foxp3+ cells (mean ± SEM).

D. Individual percentages CD4+/Foxp3+ cells of all performed CSC cultures (HD responder n=3, CLL responder n=4) with 3T40L-CLL as APC in the presence or absence of the CD70 mAb (2F2). (*p=0.0156 Wilcoxon signed rank test).
determined, since in our CSC experiments we did not observe (CD70 induced) upregulation of Bcl-2 in Treg or non-Treg CD4+ T cells (data not shown).

In addition, Treg from CLL patients seemed to have increased expression of inhibitor of apoptosis protein 1 (IAP1) (figure 5B), a gene that has been implicated in apoptotic responses to TNF\(^{25,26}\). On the other hand, we observed that Treg from CLL patients had higher expression of Fas/CD95 (figure 5D), a molecule that has been implied in activation-induced cell death in T cells\(^{27}\). Finally, Treg from CLL patients displayed lower cycling activity as assessed by the percentage of Ki-67 positive cells (figure 5E).

To test the potential functional consequences of these changes, we compared Treg from CLL patients and healthy individuals for sensitivity to drug-induced apoptosis. PBMC from CLL patients (n=6) and healthy individuals (n=6) were incubated with cytotoxic drugs or Fas-agonistic antibody and monitored for the percentage of CD25\(^{\text{bright}}\)Foxp3+ cells within the total CD4+ T cell population (figure 5F). In Treg from CLL patients we observed a strongly decreased apoptosis induction by fludarabine, Roscovitine (a drug that acts via the Noxa-Mcl1 axis\(^{24}\)) or Fas ligation when compared to Treg from healthy individuals. Together, these data support the notion that reduced apoptosis contributes to the accumulation of Treg from CLL patients.

**Discussion**

In the present study we investigated potential mechanisms behind the expansion of Treg in CLL. We observed that Treg from CLL patients as well as Treg from healthy individuals predominantly have the phenotype of primed Treg (CD4+CD25\(^{\text{bright}}\)Foxp3+CD45R0+). Although we did not find evidence for a predominant (tumor) antigen driving Treg expansion in CLL, our experiments suggest that T cell stimulation in a CLL lymph node environment might result in increased formation of Treg via CD27-CD70 costimulation. Furthermore, we observed that Treg (compared to non-Treg CD4+ T cells) have a pro-apoptotic phenotype characterized by high levels of Noxa and low expression of Bcl-2. Nevertheless, Treg from CLL patients seem to be less sensitive to apoptosis induction than Treg from healthy individuals, possibly via increased expression of Bcl-2.

Our finding that Treg in CLL patients are predominantly CD45R0+ and use a similar T cell receptor (TCR) repertoire to non-Treg CD4+ T cells is in line with a recent study which indicates that Treg arise continuously from the memory T cell pool upon antigenic stimulation\(^9\). The latter also makes it tempting to speculate about the involvement of a predominant tumor antigen/peptide in the formation of the increased numbers of Treg in CLL patients. Nevertheless, the TCR repertoire analysis performed in the current study on Treg from CLL patients did not support this possibility. Moreover, also CMV seropositivity did not influence the percentage of CD45R0+ Treg (data not shown), making a role for this antigen (which has been demonstrated to influence the CD8+ T cell repertoire in CLL\(^3\)) in the formation of Treg in CLL unlikely.
Figure 4 Apoptosis-regulating genes in Treg
CD4+ T cell populations of three healthy individuals (HD) enriched for either Treg (CD25^bright/IL7R^low) or non-Treg CD4+ T cells (CD25^-/IL7R^+) were isolated by flow cytometry (see methods) and lysed to obtain RNA and protein content.

A. relative expression levels of apoptosis-regulating genes, measured via RT-MLPA (n=3; see methods). Results of individual apoptosis-regulating genes are shown as expression relative to the total signal in the sample. Bars graph represents the mean ± SEM.

B. quantative PCR analysis of Noxa expression in the sorted cell populations. The results are presented as
To elucidate the mechanism of increased Treg frequencies in malignancies, an important question to be answered is whether tumor cells themselves can promote the expansion and activation of Tregs. Recent studies show that NHL B cells are powerful inducers of Treg\(^2\)\(^1\),\(^2\)\(^8\). Yang et al. show that CD70\(^+\) NHL B cells induce Treg formation in the lymph node via CD70 ligation. To test whether CLL cells can also be inducers of Treg, it is important to realize that CLL populations may be heterogeneous, consisting of quiescent apoptosis resistant peripheral blood cells, and actively dividing cells in lymphatic aggregates in lymph nodes and bone marrow\(^2\)\(^9\). In vitro, CD40 stimulation of CLL cells increases survival and drug resistance\(^1\)\(^3\),\(^3\)\(^0\). In vivo, there is histopathologic evidence that proliferating CLL cells are exposed to CD40L\(^+\) T cells in proliferative centers in lymph nodes\(^2\)\(^9\),\(^3\)\(^1\). After CD40 stimulation, peripheral blood CLL cells can attract CD4\(^+\)CD40L\(^+\)CCR4\(^+\) T cells by upregulating the T cell attracting chemokine CCL22 (CCL22 mRNA is constitutively expressed in CLL cells purified from involved lymph nodes)\(^3\)\(^1\). Furthermore, CD40 stimulation of peripheral blood CLL cells results in a similar apoptosis gene expression profile to lymph node CLL cells\(^2\)\(^2\). Altogether, these data suggest that CD40L stimulated CLL cells resemble CLL cells from a lymph node environment. It has been shown that in contrast to peripheral blood CLL cells, CD40L stimulated CLL cells have high CD70 surface expression\(^1\)\(^3\),\(^2\)\(^3\). We show that like CD70\(^+\) NHL cells, CD40L-stimulated CLL cells are also capable of inducing Treg in a CD70 dependent manner. CD40L-stimulated CLL cells enhanced the formation of Treg upon TCR stimulation (figure 3B and C), and this effect could be blocked by CD70 antibodies. In the absence of TCR stimulation CD40L-stimulated CLL cells were not able to induce Foxp3 expression (data not shown). These data suggest that CD27-CD70 co-stimulation may be an important step in the formation of Treg in B cell malignancies. Moreover, if indeed the increased number of Treg in CLL can be explained by CD70 ligation by CLL cells in vivo, this might also explain why these Treg have increased surface expression of CD95, since it is known that CD95 expression is upregulated on CD70 co-stimulated T cells\(^3\)\(^2\). Analysis of number and phenotype of Treg in bone marrow of CLL patients showed no difference with peripheral blood Treg (data not shown), indicating that the bone marrow is probably not the primary site of Treg formation in CLL. Altogether, we therefore hypothesize that Treg formation in CLL occurs in lymph nodes where CLL cells may function as professional APC's to induce Treg, possibly by CD27-CD70 co-stimulation. The observation that the highest frequencies of Treg are observed in CLL patients with extended disease\(^4\) (and thus with more lymph node involvement) supports this hypothesis. Moreover,

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**Enhanced formation and survival of CD4\(^+\)CD25\(^{hi}\)Foxp3\(^+\) T cells in CLL**

relative expression compared to the expression levels in Treg (black bars); the results were normalized by setting the expression levels obtained for regulatory T cells as 1.

**C.** Western blot of pooled protein lysates from Treg and non-Treg CD4\(^+\) T. Actin is used as a loading control.

**D.** Treg and non-Treg CD4\(^+\) T cells (isolated as described above) were incubated in medium (non-treated) or in the presence of CDK inhibitor Roscovitine (12.5\(\mu\)M) for 24 hours. After 24 hours viable cells were identified by flow cytometry (forward-sideward scatter).

**E.** PBMC of a healthy individual were incubated with Roscovitine (12.5\(\mu\)M) for 24 hours. Subsequently, cells were analyzed for apoptosis parameters (caspase-3 cleavage and DNA fragmentation). A Foxp3 antibody was used to identify regulatory T cells.
Figure 5 Analysis of apoptosis sensitivity of Treg from CLL patients.

CD4+ T cell populations of two CLL patients enriched for either Treg (CD25 brightly/IL7Rlow) or non-Treg CD4+ T cells (CD25/IIL7R) were isolated by flow cytometry (see methods) and lysed to obtain RNA and protein content.

A. relative expression levels of apoptosis-regulating genes, measured via RT-MLPA (n=2; see methods). Results of individual apoptosis-regulating genes are shown as expression relative to the total signal in the sample. Bars graph represents the mean; error bars indicate range.

B. RNA isolated from Treg and non-Treg CD4+ T cells (CD4) of two CLL patients (CLL) and three healthy individuals (HD) was used as input for RT-MLPA (see methods). Graphs compose 2 differently expressed genes (Bcl-2, IAP1) and one equally expressed gene (Noxa) between Tregs of healthy controls and CLL.
patients. The results are plotted as relative expression, which is calculated as follows: expression_{Treg population}/ expression_{non-Treg CD4+ T cell population}. Gene expression in non-Treg CD4+ is set as 1. Bars represent the mean relative expression; error bars indicate range.

C. Bcl-2 staining of PBMC from CLL patients (n=21) and healthy individuals (n=11). CD4 = CD3+/CD4+/Foxp3-; Treg = CD3+/CD4+/Foxp3+. Data are presented as MFI; samples were standardized by using an isotype-matched control antibody.

D. CD95 expression on T cells from CLL patients (n=19) and healthy individuals (n=5). Data are presented as MFI; samples were standardized using an isotype-matched control antibody.

E. Percentage of Ki-67+ cells in Treg and non-Treg CD4 from CLL patients (n=19) and healthy individuals (n=10). Cutoff for Ki-67 staining was determined using an isotype-matched control antibody.

F. PBMC from CLL patients (n=6) and healthy individuals (n=6) were incubated with various concentrations of fludarabine, Roscovitine and Fas-agonistic antibody CH-11 ($\alpha$-Fas). After 24 hours, cells were harvested and analyzed by flow cytometry. Finally, the percentage of Treg that remained compared to non-treated samples was calculated (see methods). Data are presented as mean ± SEM.

Extended disease may distort lymph node architecture and result in decreased niches for Tregs, resulting in Treg spill from lymph nodes into peripheral blood.

In conditions of immune stimulation, Foxp3 might not be a good marker for Treg cells, since it can be expressed by both regulatory and activated effector T cell populations. Foxp3 induction by CD40L-stimulated CLL cells might therefore be a reflection of activation rather than induction of functional Tregs. Distinguishing Treg from activated T cells in our experiments however is complicated, given the fact that Treg markers as CD25 and Foxp3 are common activation markers as well. However, for Treg induction by CD70+ NHL cells it has been shown that Foxp3 expression remained stable up to 14 days, while CD25 expression was transient. Long-term maintenance of Foxp3 expression after activation would correlate with suppressive capacity. To further investigate whether CD40L-stimulated CLL cells are capable of inducing functional Tregs that have inhibitory capacity, new and preferably unique Treg surface markers need to be found.

Next to increased formation in lymph nodes, our data also suggest that Treg in CLL may accumulate via decreased sensitivity to apoptosis. The latter may strongly influence the rapid turnover of Treg in vivo, which according to our findings seems to be facilitated by an altered balance between two molecules involved in apoptosis regulation: Noxa and Bcl-2. Therefore, the observed increased expression of anti-apoptotic Bcl-2 in CLL Treg may counterbalance the high expression of pro-apoptotic Noxa. In our drug sensitivity assays (figure 5F) we show that Treg from CLL patients are less sensitive to fludarabine, Roscovitine and CD95 ligation (the mechanism via which Treg are thought to be eliminated in vivo). The increased expression of the potent anti-apoptotic protein Bcl-2 seems to protect CLL Treg not only against p53-dependent apoptosis by fludarabine, but also p53-independent apoptosis by Roscovitine or CD95 ligation. Indeed, overexpression of Bcl-2 or knockdown of the BH-3 only pro-apoptotic protein Bid has been shown to protect against CD95L induced apoptosis in pancreatic islets cells of mice, indicating that in death receptor induced apoptosis the mitochondrial pathway can be involved.
Increased frequencies of Treg occur in many types of cancer\textsuperscript{36-38}. There is evidence that the presence of Treg in the tumor microenvironment may affect antitumor responses and promote disease progression\textsuperscript{5,6,39}. This may also be the case in CLL. Thus, targeting Treg in CLL might influence the course of the disease. Interestingly, one of the drugs used in the first line treatment of CLL, fludarabine, has been reported to reduce frequencies of Treg and affect their suppressive capacity\textsuperscript{4}. It has been hypothesized that the effect of fludarabin on CLL is partially due to its effect on Treg function and frequencies. Our data however show that Treg from CLL patients are less sensitive than Treg from healthy controls to fludarabine-induced apoptosis. Moreover, in active immunotherapy it has been shown that blockade of CTLA-4 potentiates anti-tumor T-cell responses, possibly by selective targeting of antitumor Treg\textsuperscript{40,41}. Our data show that Treg from healthy controls are very sensitive to Roscovitine, a cyclin dependent kinase inhibitor that targets Mcl-1 and therefore preferably induces apoptosis in cells with high levels of its binding partner Noxa\textsuperscript{24}. Although Treg from CLL patients are less sensitive to Roscovitine than Treg from healthy controls, by selectively targeting Treg and inducing apoptosis in CLL cells\textsuperscript{42}, Roscovitine could nevertheless be a potent adjuvant drug in active immunotherapy. Alternatively, in view of the relatively high Bcl-2 expression in CLL Treg, it would also be interesting to monitor Treg frequencies and suppressive capacity in CLL patients that are being treated with oblimersen, a therapeutical Bcl-2 antisense oligonucleotide\textsuperscript{43,44}.

In conclusion, Treg in CLL patients appear to accumulate through increased formation, facilitated by CD70 ligation by tumor cells in the lymph nodes as well as by decreased sensitivity to apoptosis due to a shifted Noxa-Bcl-2 balance. Since the increased number of Treg might be considered to negatively affect the course of the disease, targeting either one of the above-mentioned mechanisms may provide additional strategies in the treatment of CLL.

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**Authorship**

Contribution: M.J., R.M., E.B.M., R.S., A.J. and A.Y., performed experiments, M.J. and R.M. analyzed results and made the figures, M.J., R.M., R.A.W. and M.H.J. designed the research, M.J. and R.M. wrote the paper.

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References


Supplemental Figure S1 Frequency of Treg (defined as CD4+/Foxp3+ cells) in context of stage of disease.

21 CLL patients were classified according to the Rai classification: Rai 0 (n=8), Rai 1 (n=5), Rai ≥ 2 (n=8). CD4+/Foxp3+ T cells were analyzed by flow cytometry. Bars graph represents mean percentage CD4+/Foxp3+ cells ± SEM.