Studies on the immune system in CLL
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Enhanced survival and increased formation of regulatory T cells in CLL

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Submitted
Increased numbers of Treg in CLL

Abstract

Recently it has been described that chronic lymphocytic leukemia (CLL) patients have increased numbers of regulatory T cells (Treg), but the cause of this expansion is unknown. 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 antigenic stimulation. We found evidence however for increased formation of Treg via CD70 costimulation. CD40 ligand activated CLL cells (a putative model of lymph node CLL cells) strongly induced the formation of Treg via CD27-CD70 costimulation.

RT-MLPA expression analysis of 34 apoptosis-regulating genes showed that in comparison to other CD4+ T cells, Treg of both healthy individuals and CLL patients had a high expression of pro-apoptotic Noxa and a low expression of anti-apoptotic Bcl-2. However, Bcl-2 levels of Treg in CLL patients were significantly higher than in healthy individuals. Finally, at the functional level, 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.
Chapter 2

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 (26;31). So far 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 (19).

Recently it has been described that CLL patients have increased numbers of CD4+/CD25bright regulatory T cells (Treg) (4), which possess normal suppressive capacity. So far, the mechanism of this Treg expansion in CLL is not known. In healthy adults, the majority of Treg are generated in the periphery (CD4+/CD25bright/Foxp3+/CD45R0+) (1) in contrast to naturally occurring Treg (CD4+/CD25bright/Foxp3+/CD45RA+) which are generated in the thymus. It seems likely that adaptive CD4+ Treg in adults are continuously produced from the memory CD4+ T cell pool (37), 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 (37) 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 (11;33). Thus, the increase of Treg in CLL might be due either to increased proliferation, decreased apoptosis or both.

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. 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 PMBC samples contained at least 90% CD5+/CD19+ cells as assessed via flow cytometry. 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.

Flow cytometry
PBMC were stained using antibodies against CD4, CD8, CD5, CD19, CD25, CD45RA or CD45R0 and CD127 (all Becton Dickinson, San Jose, CA) 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). Antibody stained cell samples were then analysed by flow cytometry.

Isolation of T cell populations
Thawed PMBC 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+/CD25\textsuperscript{bright}/CD127\textsuperscript{low}) and non-Treg CD4\textsuperscript{+} T cells (CD4\textsuperscript{+}/CD25\textsuperscript{+}/CD127\textsuperscript{+}) 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\textsuperscript{+}/Foxp3\textsuperscript{+} 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 (21). For the spec-
tratyping, 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(16). 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 PMBC 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)(34) and in the presence or absence of a blocking CD70 mAb (clone CLB-2F2)(14). 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(10). 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.
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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 naïve-memory cell population and reflected as relative expression (gene expression in Treg set as 1).

Quantitative PCR analysis of Noxa expression

20 ng of RNA extracted from sorted cell populations (see analysis of Vβ 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′-TCAGGTTCTGAGCAGAAGAG-3′ and the 18S primers 5′-GGACAACAAGCTCCGTAAGA-3′ and 5′-CAGAAGTGACGCAGCCCTCTA-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(20). 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 anti-Mcl-1 (BD Pharmingen, San Jose, CA), monoclonal anti-Noxa (Imgenex, San Diego, CA), anti-Bcl-XL (Transduction Laboratories, Lexington, KY), rabbit-anti-Bcl-2 (Alexis Biochemicals, San Diego, CA) or antiserum to β-actin (Santa Cruz Biotechnology, Santa Cruz, CA).

Drug sensitivity assays

PBMC of both CLL patients and healthy individuals were incubated with various concentrations of Roscovitine (Sigma Aldrich, St Louis, MO), fludarabine (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 (eBio-science) 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: (Foxp3\(^+\)/(CD4\(^+\))drug stimulated)/(Foxp3\(^+\)/(CD4\(^+\))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, 10mg ml\(^{-1}\) propidium iodide and 50mg 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.
**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.
**Chapter 2**

**Results**

**CLL patients have increased numbers of regulatory T cells**

In agreement with our previous studies in CLL patients we found increased numbers of CMV-specific CD8+/CD45RA+/CD27- cells(19) but no increase in the CMV-associated CD4+/CD27+/CD28- T cell population(35) (data not shown). In addition, we observed an increase in numbers of CD4+/CD25bright/CD127low T cells (figure 1A). This phenotype has been associated with Foxp3 expression and regulatory function(17;30). Indeed, counterstaining with Foxp3 antibody showed predominantly Foxp3 positive T cells within the CD4+/CD25bright/CD127low population (figure 1B), thereby confirming recent findings that CLL patients have increased numbers of regulatory T cells(Treg).

![Figure 1. Analysis of T cell populations in CLL patients.](image)

**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(37). Thus,
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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 of CLL patients (n=2) as well as the TCR repertoire recovered from Treg of healthy individuals (n=2) and compared this repertoire to that of non-Treg CD4+ T cells (combination of naïve and memory

Figure 2. T cell receptor (TCR) repertoire analysis and phenotype of Treg from CLL patients. A and B CD4+/CD25bright/IL7Rlow (Treg) and CD4+/CD25/IL7R+ (CD4; non-Treg T cells) from 2 CLL patients (CLL) and 2 healthy individuals (HD) were sorted by FACS. RNA isolated from these T cell populations was used as input for Vβ PCR assays. The figures displayed are representative for both CLL patients and both healthy individuals, respectively. A Vβ repertoire of each T cell population (specified on the left). The bands on gel represent the product of each individual Vβ PCR (see bottom of each gel). The markers on the right indicate the area in which the product for each PCR is expected. * size marker. B Spectratyping of 3 randomly chosen Vβ families. Each peak represents a CDR3 region with a certain length. On top of the picture, the different Vβ families are indicated; the sorted T cell populations are indicated on the left.
CD4 cells) from the same individuals. We observed that the complete range of Vβ genes was used in Treg of both healthy individuals and CLL patients (figure 2A). Fragment length analysis (spectratyping) of 3 randomly chosen Vβ family PCR products showed similar peak patterns for both non-Treg T cells and Treg (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 of 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(37), 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(37) (figure 2C). We observed no difference in the percentage of CD45R0 positive Treg between healthy individuals and CLL patients. Finally, we noted that CMV seropositivity did not influence the percentage of CD45R0 positive Treg (figure 2D).

**Figure 2.** PBMC from CLL patients (n=21) and healthy individuals (n=6) were stained for CD3, CD4, Foxp3 and CD45R0. The percentage of CD45R0+ cells within the Treg (CD3+CD4+/Foxp3+) and non-Treg CD4 (CD3+CD4+/Foxp3-) population is plotted. D The percentage of CD45R0+ cells within Treg and non-Treg CD4 populations of CLL patients as depicted in figure 2C, separated according to CMV serology.
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(40). In contrast to peripheral blood CLL cells, CD40 ligand (CD40L) stimulated CLL cells (which resemble CLL cells from a lymph node environment(32)) have high CD70 surface expression(16;28). 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, the 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 augmented Foxp3 expression in CD4+ T cells of a healthy individual. Moreover, this augmentation could be blocked by 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 CD70 antibodies (figure 3C).

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

Since Treg have been described to be highly susceptible to apoptosis(33), 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 of 3 healthy individuals were sorted based on IL2R and IL7R expression(30). 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). This
finding was confirmed by quantitative PCR analysis (figure 4B). Secondly, Bcl-2 expression was found to be significantly decreased in Treg as compared to non-Treg CD4+ T cells (3.02 fold decrease; p=0.01). Protein analysis subsequently confirmed the elevated expression levels of Noxa and low expres-

Figure 3. Cell Stimulation Cultures (CSC) and Foxp3 induction.
CSC were performed with 3T3 or 3T40L stimulated CLL cells (APC) and PMBC 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 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. 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). 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).
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Treg of CLL patients are 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. In line with this assumption, we found that Treg from CLL patients express higher levels of Bcl-2 than Treg from healthy individuals (figure 5A). This was confirmed at the protein level by intracellular staining (figure 5B). Although Foxp3+/CD4+ T cells of CLL patients also demonstrated high expression of Bcl-2 when compared to healthy individuals, the Bcl-2_{Treg} : Bcl-2_{non-Treg} expression ratio was higher in CLL patients (mean ± SEM: 0.79 ± 0.14) than in healthy individuals (0.62 ± 0.07; p = 0.0008). This supports the notion that Treg of 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). We were not able to elucidate the mechanism behind high Bcl-2 levels in Treg from CLL patients and we also did not observe (CD70 induced) upregulation of Bcl-2 in CD4+ T cells during CSC experiments (data not shown). In addition, Treg of CLL patients seemed to have increased expression of inhibitor of apoptosis protein 1 (IAP1), a gene that has been implicated in apoptotic responses to TNF(36,38). On the other hand, we observed that Treg of CLL patients had higher expression of Fas/CD95 (figure 5C), a molecule that has been implied in activation-induced cell death in T cells(24). Finally, Treg of CLL patients displayed lower cycling activity as assessed by the percentage of Ki-67 positive cells (figure 5D).

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=5) were incubated with cytotoxic drugs and monitored for the percentage of CD25^{bright}/Foxp3+ cells within the total CD4+ T cell population (figure 5E). In Treg from CLL patients we observed a strongly decreased apoptosis induc-
Chapter 2

A

B

C

D

E
Figure 4. Apoptosis-regulating genes in Treg. CD4+ T cell populations of three healthy individuals (HD) enriched for either Treg (CD25bright/IL7Rlow) 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). Bars graph represents the mean ± SEM. B quantitative PCR analysis of Noxa expression in the sorted cell populations. The results are presented as 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 protein lysates from Treg and non-Treg CD4+ T cells of two healthy individuals (the protein lysates of Treg from the two healthy individuals were pooled to obtain sufficient protein concentration for western blotting). Actin is used as a loading control. D Treg and non-Treg CD4+ T cells (isolated as described above) were incubated for 24 hours in the presence of CDK inhibitor Roscovitine (12.5μM). The viable cells were subsequently identified by flow cytometry (forward-sideward scatter). E PBMC of a healthy individual were incubated with Roscovitine (12.5μM) for 24 hours. Subsequently, the cells were analyzed for apoptosis parameters (caspase-3 cleavage and DNA fragmentation). A Foxp3 antibody was used to identify regulatory T cells.

Discussion

In the present study we investigated the mechanisms behind the expansion of Treg in CLL. We observed that Treg of CLL patients as well as Treg of healthy individuals predominantly have the phenotype of adaptive Treg (CD4+/CD25bright/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 of CLL patients seem to be less sensitive to apoptosis induction than Treg from healthy individuals, possibly via increased expression of Bcl-2.
Figure 5 Analysis of apoptosis sensitivity of Treg from CLL patients.
A RNA isolated from Treg and non-Treg CD4⁺ T cells (CD4) of three CLL patients (CLL) and two healthy individuals (HD) was used as input for RT-MLPA (see methods). Graphs compose a selection of 34 apoptosis-regulating genes of which expression levels were obtained. The results are plotted as relative expression, which is calculated as follows: expressionTreg population/ expressionnon-Treg T cell population. Bars represent the mean relative expression; error bars indicate range. B 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. C 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.
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D 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.

E PMBC from CLL patients (n=6) and healthy individuals (n=6) were incubated with various concentrations of Roscovitine, Fas-agonistic antibody CH-11 (α-Fas) and fludarabine. After 24 hours, the cells were harvested and analyzed by flow cytometry. Finally, the percentage of Treg that remained compared to non-treated samples was calculated.

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(37). 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, making a role for this antigen (which has been demonstrated to influence the CD8+ T cell repertoire in CLL(19)) in the formation of Treg in CLL unlikely.

Recent studies show that NHL B cells are powerful inducers of Treg(23;40). Yang et al. propose that this Treg induction takes place in the lymph node via CD70 ligation by CD70+ NHL B cells. The results from our current study suggest that in CLL this might also be the case: CD40L stimulated CLL cells (resembling CLL cells from a lymph node environment(32)) enhanced the formation of Treg upon TCR stimulation (figure 3B and C), and this effect could be blocked by CD70 antibodies. Therefore, it seems 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). Analysis of number and phenotype of Treg in bone marrow of CLL patients showed no difference with peripheral blood Treg (data not shown), which indicates that the bone marrow is probably not the primary site of Treg formation in CLL. Altogether, we consider it more likely that Treg formation in CLL takes place in lymph nodes where CLL cells may function as professional APC to induce Treg, possibly by CD27-CD70 co-stimulation. The observation that the high-
est frequencies of Treg occur in CLL patients with extended disease (4) (and thus with more lymphoid tissue) supports this hypothesis. Besides via increased formation in the 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. Previously, we demonstrated that upon T cell activation, Noxa is expressed and may play an important role in determining the size of activated T cell populations (2). Bcl-2 and Noxa are not direct binding partners (5;7), hence a direct reciprocal effect of their altered expression is unlikely. Rather, the effects on changes in apoptosis thresholds probably occur indirectly via shared binding partners, such as Mcl-1 and Bim (13). In addition, the increased expression of Bcl-2 also seems to protect CLL Treg against CD95 ligation (the mechanism via which Treg are thought to be eliminated in vivo (11)). Increased frequencies of Treg occur in many types of cancer (6;18;39). There is evidence that the presence of Treg in the tumor microenvironment may affect antitumor responses and promote disease progression (8;9;22). 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 (4). Thus, the effect of this drug on CLL might partially be contributed to its effect on Treg. Moreover, in active immunotherapy it has been shown that blockade of CTLA-4 potentates anti-tumor T-cell responses, possibly by selective targeting of antitumor Treg (15;27). Our data suggest that Treg 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 (13). By selectively targeting Treg and inducing apoptosis in CLL cells (12), Roscovitine could therefore 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 (25;29).

In conclusion, Treg in CLL patients appear to accumulate through increased
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formation, facilitated by CD70 ligation by tumor cells in the lymph nodes as well as by decreased sensitivity to apoptosis. 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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Chapter 2

Reference List


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