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IL-21 induces proliferation of CD40-stimulated CLL cells and is present in the lymph nodes from CLL patients

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Abstract

Chronic Lymphocytic Leukemia (CLL) cells accumulate in peripheral blood (PB) but proliferate in secondary lymphoid organs, where they also display increased resistance to apoptosis. The signals involved in CLL proliferation in vivo are largely unknown. In the proliferation centres (PCs), CLL B cells are in close contact with activated CD40L+ CD4+ T cells. In vitro stimulation of PB CLL cells with CD40L results in chemoresistance and an anti-apoptotic profile resembling the lymph node (LN) microenvironment, but induces little proliferation. Apart from expressing CD40L, activated T cells can also secrete IL-21, which has an essential role in providing help to normal B cells. Here, we dissected the signals provided by activated T cells versus CD40-stimulation to CLL with respect to proliferation and drug resistance. In vitro stimulation of PB CLL cells with CD40L+IL-21 resulted in proliferation of CLL cells. Surprisingly, IL-21 abrogated the resistance induced by CD40L to certain cytotoxic drugs but not to the BH3-mimetic ABT-737, which might be related to blockade of the CD40L-dependent increased in Bcl-XL expression. In addition, microarray analysis showed that 51 genes were differentially regulated by IL-21, among which were components of the JAK-STAT (STAT3), and apoptosis pathways (Bcl-XL) as well as granzymeB (GZMB). This “IL-21/CD40L/CLL” gene signature could also be detected in CLL cells cultured with autologous activated T cells and in LN samples from CLL patients. Finally, we could demonstrate the presence of IL-21 in LN samples from untreated patients. These results suggest that activated T cells from CLL patients can provide strong CD40L and IL-21 stimulation to CLL cells in vivo, which is not only relevant for understanding the biology of CLL but might also open new treatment venues.
Introduction

Chronic lymphocytic leukemia (CLL) is a hematological disorder characterized by the progressive accumulation of mature, monoclonal CD5+ B lymphocytes in peripheral blood (PB), bone marrow (BM), and secondary lymphoid organs such as the lymph nodes (LN). CLL cells relentlessly accumulate in vivo, due both to increased resistance to apoptosis and proliferation (1). Given the fact that circulating CLL cells are largely arrested in the G0/G1 phase of the cell cycle and rapidly undergo spontaneous apoptosis in vitro (2), both processes are believed to be governed by the interaction of CLL cells with the microenvironment present in lymphoid organs (3). Several surface and soluble molecules from this protective microenvironment, such as SDF-1, BAFF, APRIL (4-6) and CD40 ligand (CD40L) (7, 8), have been demonstrated to increase resistance to spontaneous and drug-induced apoptosis of CLL cells, but much less is known about the mechanisms of proliferation of CLL cells.

In the proliferation centers present in the LN, CLL cells are in contact with activated CD40L+ CD4+ T cells (9) and it has been proposed that these cells can support the growth of CLL cells through CD40 ligation. In fact, autologous activated CD4+ T cells have been shown to play a key role in CLL expansion in a recently developed adoptive transfer mouse model of CLL (10). However, in our experience, although strong CD40L stimulation alters the apoptotic profile of CLL cells and increases their resistance to apoptosis (7), it only induces minimal proliferation. Activated CD4+ T cells can also produce IL-21, a cytokine that has a fundamental role in the development of T cell-dependent B cell responses.

IL-21 shares the common receptor γ-chain with IL-2, IL-7 and IL-15, and is produced by activated human CD4+ T cells, as well as Th17, T follicular helper and NKT cells (11). Depending on the interplay with co-stimulatory signals and on the developmental stage of a B cell, IL-21 can induce proliferation, differentiation into Ig-producing plasma cells, or apoptosis in both mice and humans (12, 13). In CLL cells, direct stimulation with IL-21 led to Bim-dependent apoptosis (14). IL-21 treatment also led to apoptosis of CLL cells if administered after CD40L stimulation (15). However, the distinctive presence of activated CD40L+ T cells in PCs from CLL patients led us to hypothesize that, in the lymphoid tissue microenvironment, CLL cells could be exposed simultaneously to CD40L and IL-21 and that this could be in part responsible for their proliferation. In this work, we wished to study the regulation of CLL cell apoptosis and proliferation by IL-21 in CLL cells.

Materials and Methods

Patient samples

Patient material (PB, BM aspirates and LN biopsies) was obtained from CLL patients (diagnosed according to the NCI-WG guidelines), after informed consent, during routine
follow-up or diagnostic procedures at the Department of Hematology of the Academic Medical Center (AMC), Amsterdam. The studies were approved by the Ethical Review Board of the institution and conducted in agreement with the Helsinki Declaration of 1975, revised in 1983. PB mononuclear cells (PBMCs) of CLL patients and healthy donors (HD), and BM-derived mononuclear cells were isolated by Ficoll density gradient centrifugation (Pharmacia Biotech, Roosendaal, the Netherlands) and stored in liquid nitrogen. LN material was minced to isolate cells. Expression of CD5 and CD19 (both Beckton Dickinson (BD) Biosciences) on leukemic cells was assessed by flow cytometry (FACScalibur, BD Biosciences) and analyzed with CellQuest software (BD Biosciences). All samples contained at least 90% CD5+/CD19+ cells as assessed via flow cytometry. During all in vitro experiments, cells were maintained in culture medium: Iscove’s modified Dulbecco medium (IMDM, Gibco, Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100μg/ml gentamycin and 0.00036% β-mercaptoethanol.

Reagents
Fludarabine (F-Ara-A), Roscovitine and Propidium Iodide (PI) were purchased from Sigma-Aldrich Chemie B.V. ABT-737 was obtained under MTA from Abbott (Abbott Park, courtesy Dr. S. Rosenberg). Bortezomib was obtained from Janssen-Cilag (Tilburg, The Netherlands).

Assessment of proliferation and apoptosis
PBMC from CLL patients were thawed and labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes). For this, cells were resuspended in PBS at 1,0 x 10^7/mL in 0,5 μM CFSE for 8 min at 37°C and then washed in IMDM containing 10% FCS. After labelling, cells were diluted to a concentration of 2,5 x 10^5/mL and cultured with CD40 ligand (CD40L)-transfected NIH3T3 cells (3T40L) as described previously (7) or the control cell line NIH3T3 (3T3), in the absence or presence of recombinant human IL-21 (25 ng/ml unless otherwise stated, Cat# PHC0214, Gibco, Invitrogen) or CpG (1,5 μg/mL, ODN2006, Invivogen). After 5 days, proliferation was assessed in a FACS Canto (BD Biosciences) and analyzed with FlowJo software (TreeStar). Apoptosis was analyzed in the same cultures by evaluation of mitochondrial membrane potential and membrane integrity, with DioC6 (Molecular Probes) and propidium iodide (PI; Sigma-Aldrich Chemie B.V.) respectively. Stained cells were analyzed in FACS Calibur flow cytometer (BD Biosciences).

Drug sensitivity assay
PBMC from CLL patients were cultured with 3T40L or 3T3 cells, in the presence or absence of IL-21 for 3 days. Then, cells were removed from the wells, washed and placed in 96-well plates with growing concentrations of cytotoxic drugs for 24-48 hours. Cell viability was assessed by DiOC6/PI staining, as stated before.
Western Blot
Western blotting was performed as described previously (16). Blots were probed with polyclonal rabbit anti-human Bcl-X_L (Catalogue #620211, BD Biosciences), polyclonal rabbit anti-human Bim (Catalogue #Aap-33E, Stressgen), polyclonal rabbit anti-human Mcl-1 (Catalogue #4572, Cell Signaling), monoclonal mouse anti-human Noxa (Catalogue #IMG-349A, Imgenex), polyclonal goat anti-human β-actin (clone I-19; Santa Cruz Biotechnology) and monoclonal mouse anti-human Tubulin (Catalogue #T6199, Sigma-Aldrich Chemie B.V.).
IRDye 680 donkey anti-rabbit IgG, IRDye 800 donkey anti-goat IgG or IRDye 800 donkey anti-mouse IgG (Westburg, Leusden, the Netherlands) were used as secondary antibodies. Western blots were scanned on the Odyssey imager (LI-COR Biosciences), and subjected to densitometry with the Odyssey software (Odyssey Application software version 3.0).

Sample preparation for microarray analysis
PBMC from CLL patients were thawed and resuspended at a concentration of 1.6 x 10^6/mL and cultured with 3T40L, in the absence or presence of recombinant human IL-21 (25 ng/ml) for 16 hours. For stimulation with autologous activated T cells, CLL cells were positively selected with CD19^+ magnetic beads (MACS, Miltenyi Biotec). After selection, the percentage CD5^+CD20^+ CLL cells, and CD3^+ T cells (in the flow-through fraction), were determined by flow cytometry. CLL cells and T cells were subsequently added in a 1:2 ratio (T:B) in the presence of CD3 (clone 1XE, CLB, Amsterdam, The Netherlands) and CD28 (clone 15E8, Sanquin, Amsterdam, The Netherlands). Cells were stimulated 16 hours at 37ºC in a 5% CO2 incubator. Mononuclear cells from LN and BM were thawed together with the PBMCs and kept at 4ºC for the same period of time. The following day, cells were harvested on ice, stained with APC-conjugated anti-human CD5 and PerCP Cy5.5-conjugated anti-human CD19 or FITC-conjugated anti-human CD20 (all BD Biosciences). CD5^+CD19^+ or CD5^+CD20^+ cells were sorted in a FACS Aria (BD Biosciences). Purity was > 95% in all cases. Immediately after sorting, cells were washed with PBS, resuspended in Trizol (Invitrogen) and stored at -80ºC. RNA was extracted according to the Trizol’s manufacturer instructions and then cleaned with RNeasy kits (Qiagen) to obtain high purity RNA. The concentration and purity of the RNA were determined with a NanoDrop spectrophotometer ND-1000 (NanoDrop Technologies) and the ribosomal RNA band integrity was determined on an Agilent 2001 BioAnalyzer® (Agilent Technologies). RNA samples used for microarray experiments met all of the following criteria: A260/A280 ratio >2.0, A260/A230 ratio >1.8 and a sharp distinction at the small site of both the 18S and 28S ribosomal RNA bands/peaks.
Microarray data analysis
Fragmentation of cRNA, hybridization to HG-U133 Plus 2.0 microarrays (Affymetrix) and scanning was carried out according to the manufacturer’s protocol at the Microarray Department of the University of Amsterdam (MAD, Amsterdam The Netherlands). Intensity values and P-values for determining significant regulation (p<0.0025) were assigned with GCOS software (Affymetrix) and normalized to an average intensity of 100 using the MASS5.0 algorithm (Affymetrix). Microarray data have been deposited at the GEO database at the NCBI website under number [to be disclosed]. The data was normalized with the GCOS program using the mas5 algorithm, normalized to a trimmed mean of 100. In-built differential gene expression algorithms were applied between groups with the R2 program (Department of Oncogenomics, AMC, Amsterdam, the Netherlands).

Reverse transcription - Multiplex Ligation-dependent Probe Amplification (RT-MLPA) assay
Reverse transcription-multiplex ligation-dependent probe amplification assay (RT-MLPA) procedure was performed as described previously (17). Briefly, 150 ng total RNA as obtained from sorted 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; 32 cycles, 20 sec at 95°C, 30 sec at 60°C and 1 min at 72°C) using one unlabelled 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). Results were analysed 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 software (Microsoft). Data was normalised by defining house-keeping (HK) genes with Genorm program, as previously described (18) and expressing individual peaks relative to the sum of expression values of the HK genes.

IL-21 qPCR
PBMCs from healthy donors or CLL patients were cultured in medium, in the presence of agonistic antibodies against CD3 (1 μg/ml, clone 1X1) and CD28 (3 μg/ml) for 24 hours or PMA (100 ng/ml, Sigma-Aldrich Chemie B.V.) and Ionomycin (1 μg/ml, Sigma-Aldrich Chemie B.V.) for 6 hours. Then, cells were washed and lysed with Trizol (Invitrogen). Total RNA was extracted according to manufacturer’s instructions. Oligodeoxythymidine (oligo dT) primed cDNA was synthesized using RevertAid H Minus Reverse Transcriptase (Fermentas). From these cDNA pools, IL-21 was amplified by polymerase chain reaction (PCR) with Fast SYBR Green Master Mix on a StepOne Plus machine (Life Technologies) using the following primers: IL-21 forward 5’-GGCAACATGGAGAGGATTG-3’ and
IL-21 reverse 5’-AAGCAGGAAAAAGCTGACCA-3’. The results were normalized to 18S using the ΔCt method.

Immunohistochemistry
Paraffin-embedded LN samples from CLL patients, obtained from the Department of Pathology (AMC, Amsterdam, the Netherlands), were cut, deparaffinised and rehydrated through xylene and ethanol. Endogenous peroxidase was blocked by incubation in 0.03% H₂O₂ in methanol, for 20 min at RT, and then heat-induced epitope retrieval was performed in Tris-EDTA (pH= 9.0) buffer. Samples were incubated with a rabbit anti-human IL-21 antibody (Catalogue #14-6465, eBioscience) overnight at 4°C. Immunoreactive cells were visualized using BrightVision Poly-HRP-Anti-mouse/rabbit/rat IgG and BrightDAB (Immunologic, the Netherlands), according to the manufacturer’s instructions, and lightly counterstained with hematoxylin. Isotype control sections were prepared under identical immunohistochemical conditions, replacing the primary antibody with a purified, normal rabbit IgG control antibody (Dako).

Statistical analysis
Differences between groups were compared using either the Student’s t test or the Mann-Whitney U test (paired, when applicable). P-values < 0.05 were considered statistically significant.

Results
IL-21 induces both proliferation and apoptosis in CLL cells
We first investigated whether IL-21 could induce apoptosis in the presence or absence of CD40L. For this, CLL cells were cultured with a control cell line or CD40L-expressing cells and different concentrations of IL-21. After 5 days, apoptosis was measured by DiOC6/PI staining. As shown in Figure 1A, IL-21 induced significantly higher levels of apoptosis in unstimulated CLL cells when compared to CD40-stimulated CLL cells.

We then studied the ability of IL-21 to induce proliferation of CLL cells and whether CD40 stimulation was necessary for the induction of proliferation. For this, CLL cells were stained with CFSE and cultured on a feeder layer of cells expressing CD40L or the control cell line, together with different concentrations of recombinant human IL-21. After 5 days, CFSE dilution was assessed and the percentage of divided cells was evaluated. As depicted in Figure 1B, IL-21 induced proliferation of CLL cells even at the lowest concentration but only when in combination with CD40L. Next, the level of proliferation induced by IL-21 was compared with that induced by CpG, which has already been reported as a strong proliferation signal for CLL cells (19, 20). Figure 1C shows that a proportion of CLL samples proliferated in response to CD40L, which is in line with previous reports, and this was increased both by IL-21 or CpG. Both stimuli proved to be equally efficient.
in this sense. When we analyzed the division index of these samples (Figure 1D), which is the average number of cell divisions that a cell in the original population has undergone, we found that number to be lower than 2 for all treatments. This meant that CLL cells had undergone less than 2 divisions. To check whether this was due to shortage of nutrients/stimulation or a block in proliferation, we cultured CFSE-stained CLL cells in the presence of CD40L-expressing cells and IL-21 and renewed the stimuli together with fresh medium

Figure 1. The balance between IL-21-induced apoptosis versus proliferation depends on CD40 stimulation. A. CLL cells were cultured at 0.25x10^6 cells/ml, with a control cell line or with CD40L-expressing fibroblasts in the absence or presence of different concentrations of IL-21. Cell death was assessed after 5 days by DiOC6/PI staining. Results are shown as the % of cell death (DiOC6− PI+/-), mean±s.e.m, n=4; B. CFSE-stained CLL cells were cultured as in C&D. The % of divided cells was calculated with FlowJo program. Results are depicted as mean±s.e.m, n=3; C & D. CFSE-stained CLL cells were cultured with CD40L-expressing fibroblasts in the absence or presence of IL-21 (25 ng/ml) or CpG (1,5 μg/ml). Proliferation was assessed after 5 days and C. the % of divided cells and D. division index were calculated with FlowJo program. Results are depicted as individual values for 9 patients, together with the average value; E. CFSE-stained CLL cells were cultured with CD40L-expressing fibroblasts in the absence or presence of IL-21 (25 ng/ml) and the stimulation was renewed after 3 and 7 days. Proliferation was assessed at day 3, 7 and 10. Results are shown as representative histograms for 2 patients.
every 3-4 days. This continuous supply of stimuli and nutrients led to proliferation of the majority of CLL cells (Figure 1E). Although the extent of CFSE dilution did not allow for precise quantification of the division index, it was clear from the CFSE histograms that the cells had undergone at least 3 divisions. These results clearly indicate that IL-21 is able to induce sustained proliferation of CLL cells when acting in concert with CD40 stimulation.

IL-21 alters the anti-apoptotic prolife induced by CD40L and abrogates resistance to certain cytotoxic drugs

CD40 stimulation of CLL cells leads to decreased spontaneous apoptosis as well as resistance to many cytotoxic drugs and this can be explained by the CD40-dependent up-regulation of anti-apoptotic molecules, such as Bcl-X<sub>L</sub>, Bfl-1, XIAP and Mcl-1 and down-regulation of pro-apoptotic mediators, such as Bim and Noxa (7, 16, 21). We next wished to examine whether IL-21 could affect drug resistance induced by CD40. CLL cells (0.25x10<sup>6</sup> cells/ml) were cultured for 72 hours in the presence of a control cell line, CD40L-expressing fibroblasts or CD40L-expressing fibroblasts together with IL-21, and then cells were washed and re-seeded in the presence of increasing concentrations of cytotoxic drugs for additional 48 hours. Cell death was assessed by DiOC6/PI staining. As shown in Figure 2A-D, sensitivity of CLL cells to fludarabine, roscovitine, bortezomib and the BH3-analog ABT-737 was strongly reduced after CD40 stimulation. Simultaneous incubation of CLL cells with CD40L+IL-21 differentially affected the development of drug resistance. Resistance to fludarabine was completely reversed (Figure 2A), while resistance to roscovitine and bortezomib was affected to a lesser extent (Figure 2B and C, respectively) and resistance to ABT-737 was almost unaffected (Figure 2D).

To unravel the mechanism behind this differential modulation of drug resistance, we explored the balance between pro- and anti-apoptotic mediators in CLL cells stimulated with CD40L and/or IL-21. CLL cells were cultured together with a control cell line or CD40L-expressing cells, in the absence or presence of IL-21, for 16 hours and then sorted. The effect of the different treatments on the transcription of pro- and anti-apoptotic genes was monitored with a multiplex assay able to simultaneously quantify expression of several apoptosis-related genes. As described previously, in vitro CD40 stimulation of CLL cells induced the transcription of Bcl-X<sub>L</sub> and led to a decrease in the expression of Bim, while Mcl-1 and Noxa mRNA levels were not significantly modified (Figure 2E-H). Of all the genes studied, Bcl-X<sub>L</sub> was the only one that was significantly different between CD40L and CD40L+IL-21 stimulation. Namely, IL-21 significantly impaired CD40L-induced upregulation of this gene (Figure 2E and Supplemental figure 1).

These results were confirmed by immunoblot on cell extracts obtained after 72 hours. As shown in Figure 2I, protein levels of Bcl-X<sub>L</sub> increased in CLL cells after CD40 stimulation, but this was impaired in the presence of IL-21. On the other hand, the CD40L-dependent decrease in Bim protein levels was not affected by the cytokine. Protein levels of Noxa decreased in CD40L-treated samples and this was partially reverted by IL-21, while
Mcl-1 levels remained constant at this time point. This led to an increased Noxa/Mcl-1 ratio in samples treated with CD40L+IL-21 compared to CD40L alone (Figure 2I and Supplemental figure 2).

In summary, results depicted in this section show that IL-21 modifies the anti-apoptotic profile induced by CD40 stimulation in CLL cells, by impairing the increase in Bcl-XL levels, and makes CLL cells more susceptible to certain cytotoxic drugs.

IL-21-induced gene signature in CD40-stimulated CLL cells can also be induced by activated T cells

Next, we wished to find evidence of IL-21 signaling in vivo in CLL patient samples and decided to investigate this indirectly, through the detection of an IL-21 gene expression signature of CD40-stimulated CLL cells (“IL-21/CD40L/CLL” gene signature).

For this purpose, freshly thawed CLL PBMCs were incubated overnight on CD40L-expressing cells in the presence or absence of IL-21. Then, CD19^+ CD5^+ CLL cells were sorted and RNA was purified to analyze gene expression in Affymetrix whole genome microarrays. Analysis revealed that 51 genes were significantly regulated by IL-21 in CD40-stimulated cells (p<0.01, fold change>3, Figure 3A and Supplemental Table 1). Of these, 17 genes were up-regulated and 34 down-regulated. The most striking difference was the up-regulation of Granzyme B by IL-21 in CD40-stimulated CLL cells, which in is line with previous studies where IL-21 has been shown to induce granzyme B expression in healthy B cells and CLL cells (22-24). To validate these observations, we evaluated Granzyme B RNA expression by MLPA and found that indeed, IL-21 induced up-regulation of this gene in sorted CLL cells, independently of CD40 stimulation (Figure 3B), but the level of induction showed great variation between patients and did not reach statistical significance. Additionally, microarray analysis revealed a significant decrease in Bcl-X₇ after IL-21 stimulation of CD40-stimulated cells (Figure 3A), which confirms our previous observations at both mRNA and protein level (Figure 2E & I).

To validate this “IL-21/CD40L/CLL” gene signature, we generated an “IL-21 score” which was calculated as the average absolute fold induction of all the genes modulated by IL-21, compared to their levels in matched PB samples. As expected, this score was 5
Figure 3. IL-21 gene expression signature of CD40-stimulated CLL cells is increased in CLL cells cultured with autologous activated T cells and in lymph nodes from CLL patients. Gene expression analysis (U133 plus 2.0 array, Affymetrix) of sorted CD20+CD5+ CLL cells, cultured with CD40L-expressing fibroblasts in the absence or presence of IL-21 for 16 hours; A. Heatmap of 51 differentially expressed genes (anova \( p < 0.01 \); fold change > 3; min. present calls > 3; min. highest expression > 200), depicting results for paired samples from 4 patients; B. Expression levels of Granzyme B (GZMB) analyzed by MLPA in the same samples as in Figure 2E-H. Results are shown as relative expression to housekeeping (HK) genes; C-F. An IL-21 score was computed as the average of the fold increase in mRNA expression level of the 51 genes shown in A, in: C. cells cultured with CD40L-expressing fibroblasts compared with CD40L-expressing fibroblasts+IL-21 (25 ng/ml), or D. with activated T cells (Tact); or in bone marrow and LN from E. our patients or F. from published data, relative to their corresponding level in PB. P values shown were obtained by Mann-Whitney test.
Figure 4. IL-21 expression in lymph nodes from CLL patients. A & B. IL-21 expression was studied by qRT-PCR in RNA from: A. PBMCs from healthy donors and CLL patients, after activation with a-CD3 +/- aCD28 antibodies or PMA+Ionomycin, or B. total frozen LN samples from CLL patients; C. IL-21 expression was analyzed in paraffin-embedded LN samples from 2 CLL patients by immunohistochemistry.
times higher in CLL cells after stimulation with CD40L+IL-21 (median = 9.4, range = [4.8-30.4], n=4), compared to that obtained for CD40-stimulated cells (median= 1.7, range = [1.0-2.1], n=4, Figure 3C). Then, we calculated the same score in microarray data from CLL cells that were co-cultured with autologous activated T cells and compared it to the score in matched CD40-stimulated CLL cells. Previous experiments revealed that activated T cells from CLL patients induced proliferation of CLL B cells via a CD40L- and IL-21-dependent mechanism (Tromp et al, chapter 3, this thesis). Indeed, the “IL-21 score” obtained from CLL B cells cultured with autologous activated T cells had a median of 8.2 (range = [2.8-61.8], n=5) whereas, in matching CD40-stimulated samples, a median score of 1.4 was observed (range = [1.2-1.8], n=5, Figure 3D). These results indicate that IL-21 signaling could be detected through this gene expression signature in a more physiological context.

Finally, we investigated whether the “IL-21/CD40L/CLL” signature was present in LN and BM samples from CLL patients. We obtained RNA from sorted CLL cells from 5 BM and 2 LN samples from our patient cohort, and calculated the score in comparison to their matched PB samples. In this case, the values obtained were lower than those observed in the in vitro systems. For the BM samples the median obtained was 1.1 (range = [1.0-1.2]). In the case of the LN samples, we obtained scores of 1.5 and 1.6, which was suggestive of IL-21 presence but inconclusive, due to the low number of samples (Figure 3E). To investigate this issue further, we interrogated an available dataset generated by Herishanu et al (25). These authors obtained RNA from sorted CLL cells from matched PB, BM and LN samples and performed microarray analysis using the same platform. The raw data was obtained from their public deposit and then we generated the “IL-21 scores”, which are depicted in Figure 3F. As can be seen, most of the LN samples presented scores higher than those found for the paired BM samples. Collectively, the data presented in this section are indicative of IL-21 signaling in CLL in vivo and suggests that this signature is higher in the LNs of CLL patients.

IL-21 is present in lymph nodes from CLL patients

Next, we explored whether we could directly detect production of IL-21 in activated CLL T cells and in LN samples from CLL patients. First, we measured IL-21 mRNA by qRT-PCR in RNA obtained from CLL or healthy PBMCs stimulated with anti-CD3 ± anti-CD28 agonistic antibodies or PMA /Ionomycin. For all stimulations, we could detect similar levels of IL-21 induction both in healthy and CLL samples, compared to the control condition (Figure 4A). Subsequently, RNA was obtained from frozen LN samples of CLL patients and a similar analysis was performed. In these samples, we also could also detect IL-21 mRNA, albeit at variable levels (Figure 4B). These results were confirmed by immunohistochemistry in paraffin-embedded LN samples, where we could detect the presence of IL-21+ cells (Figure 4C).
In this work, we describe for the first time that IL-21 can induce proliferation of CLL cells when administered simultaneously with CD40L, to levels similar to those found for CD40L+CpG – a combination that was shown to be a potent inducer of proliferation in CLL cells in vitro (19). The combined stimulation of CLL cells with CD40L and IL-21 could reflect the in vivo lymph node microenvironment where activated T cells and CLL B cells have been proposed to interact (2, 3).

The discrepancy between our results and those published before is easily explained by the differences in stimulation. Previous work reporting apoptosis induction by IL-21 employed the cytokine alone on freshly isolated CLL cells (14) or in CLL cells stimulated with CD40L, which were then removed from this stimulation (15, 26). It has been shown for murine B cells that IL-21 can primarily induce apoptosis if administered together with LPS or CpG, but instead promotes proliferation when combined with anti-IgM and/or anti-CD40 agonist antibodies (12, 13). In our experiments, IL-21 also induced apoptosis of CLL cells when administered alone but concomitant CD40L-signaling shifted the balance from apoptosis to proliferation. Based on the pro-apoptotic role of IL-21 on CLL cells, some authors have proposed the initiation of combination studies of IL-21 with fludarabine- and rituximab-based therapy in CLL (14, 15, 26). In this sense, our results suggest that while this type of therapy could potentially eliminate some CLL cells, it could also induce the proliferation of those CLL cells in contact with activated T cells, which would exacerbate disease progression.

We have described that stimulation of CLL cells with a CD40L-expressing cell line leads to an anti-apoptotic profile and increased resistance to cytotoxic drugs (7). Here, we have shown that the addition of IL-21 during stimulation of CLL cells with CD40L alters the anti-apoptotic balance, by interfering with the induction of Bcl-X\textsubscript{L} and increasing the level of Noxa, tipping the balance towards apoptosis. These alterations are accompanied by an increase in the susceptibility to certain cytotoxic drugs. Interestingly, resistance to the BH3-mimetic ABT-737 was relatively unaffected by the addition of IL-21. ABT-737 is very effective against Bcl-2 and Bcl-X\textsubscript{L}, but does not bind to Mcl-1 or A1/Bfl-1 (27, 28). As reported before, CLL cells are quite sensitive to ABT-737, but upon stimulation with CD40L this is reduced approximately 100-fold (29). We and others (27, 28) have argued that in the concentrations used in vitro, ABT-737 is able to completely neutralize Bcl-2 and Bcl-X\textsubscript{L}, even after their CD40L-induced increase, and therefore sensitivity will depend on the levels of Mcl-1 and/or Bfl-1/A1 in the cells. These results acquire relevance in the in vivo situation. If this scenario were representative of the situation in LN, this would imply that proliferating CLL cells are more susceptible to certain drugs than the bulk of resting cells, which are protected by interaction with stromal cells. Support for this hypothesis could come from interpretation of former clinical trials on elimination of residual disease or from examination of LN from patients treated with fludarabine. It would be expected
that in responsive patients, the amount of proliferation centers would be decreased after therapy. If this increased susceptibility of proliferating cells could indeed be confirmed, it could be useful in the design of future combination therapies.

An important point of this work was to ascertain whether we could find evidence of IL-21 presence/signaling \textit{in vivo}. For this, we employed different strategies. First, we attempted an indirect strategy, which has been used to detect BCR signaling in LN from CLL patients (25). We generated an \textit{in vitro} “IL-21/CD40L/CLL” signature by evaluating gene expression changes induced by IL-21 in CD40-stimulated CLL cells. We could detect 51 genes that were differentially regulated by IL-21 in this context. Of note, Granzyme B was found to be up-regulated by IL-21 in sorted CLL cells, both by microarray analysis and MLPA. Although this observation could be the result of a small number of contaminating CD8+ T or NK cells, production of Granzyme B in IL-21-treated purified CLL and healthy B cells has already been reported by another group (22-24). Interestingly, while IL-21 has been proposed to endow B cells with cytotoxic activity, we did not detect expression of other granzymes (microarray analysis) or perforin (microarray and MLPA, Supplemental figure 1). The biological significance of Granzyme B up-regulation by IL-21 in CLL cells remains to be elucidated.

The generated gene signature was used to interrogate a more physiological \textit{in vitro} system than the commonly used CD40L transfectant system. Indeed, here we found evidence of IL-21 signaling in CLL cells cultured with autologous activated T cells. This finding is in line with previous evidence that pointed to a role of IL-21 in the proliferation of CLL cells induced in this system. The “IL-21 score” induced by activated T cells was comparable to that achieved by the combination of recombinant human IL-21 and CD40L-expressing cells. Next, we investigated “IL-21 scores” in the lymphoid compartments from CLL patients. Both in samples from our cohort of patients and in data available from a previous publication (25), we found a small but consistent increase in these scores compared to those found in BM samples. The low scores are to be expected since RNA samples were obtained in both cases from the total CLL B cell population of these organs, and the amount of cells potentially in contact with IL-21 could be small. It is difficult to estimate the size of this sub-population of CLL cells. One approximation could be to consider the total proliferating cells as potentially influenced by the cytokine. In that sense, Herishanu et al (25) have shown that Ki-67+ cells represent between 3 and 8% of the CD3- cells in the LN. It is therefore highly relevant that we could find significant differences in the “IL-21 score”. To further strengthen this point, we evidenced the presence of IL-21 in LN samples both by qRT-PCR and IHC. An important point that remains to be demonstrated is whether the activated CD40L+ T cells that are present in PCs are responsible for the production of IL-21 in CLL LN.

Due to its relatively slow clinical progression, CLL was classically described as a disease of accumulation rather than proliferation. It has recently become evident that there is substantial proliferation within CLL cells and that high levels of proliferation of the
leukemic population are correlated with worse prognosis (30). Recently, the circulating Ki-67 index in plasma has been shown to have significant correlation with lymph node involvement and Rai stage and high values of this index were significantly associated with shorter survival (31). In this work, we have shown that stimulation with CD40L and IL-21 is sufficient to induce proliferation of CLL cells and also found strong suggestions of IL-21 signaling in LN from CLL patients. This would suggest that therapies aimed at blocking IL-21 signaling, in combination with other cytotoxic drugs, could help decrease CLL burden. This type of immunotherapy would be the first to exclusively address blockade of proliferation in CLL.
Reference List


### Supplemental table 1: Gene signature of IL-21 in CD40-stimulated CLL cells

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Supplemental Figure 1. Analysis of mRNA levels of pro-/anti-apoptotic mediators in CLL cells treated with IL-21 and/or CD40L. CLL cells were cultured with a control cell line, IL-21 alone (25 ng/ml), CD40L-expressing fibroblasts alone or the combination of these stimuli for 16 hours. Then, CD20+ CD5+ cells were sorted, RNA was extracted and the expression of different apoptotic mediators was analyzed by MLPA. Results are shown as mean (±s.e.m., n=4), as relative expression to housekeeping (HK) genes.

Supplemental Figure 2. Quantification of protein levels of Noxa and Mcl-1 in CLL cells treated with IL-21 and/or CD40L. A & B. Protein levels of A. Noxa and B. Mcl-1 were measured by quantification of WB specific bands relative to the expression of β-actin or tubulin, in CLL PBMCs treated with IL-21 and or CD40L for 72 hrs, n=2-4; C. Noxa/Mcl-1 ratio was calculated for each sample by dividing the intensity of Noxa expression by that of Mcl-1.