IL-21 and CD40L signals from autologous T cells can induce antigen-independent proliferation of CLL cells

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Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the progressive accumulation of mature, monoclonal CD5+ B lymphocytes in peripheral blood (PB), bone marrow (BM), and lymph nodes (LN). CLL cells accumulate because of proliferation and resistance to apoptosis.1 Both processes are believed to be governed by the interaction of CLL cells with the microenvironment in lymphoid organs,2 because circulating CLL cells are largely arrested in the G0/G1 phase of the cell cycle and undergo spontaneous apoptosis in vitro.3 Various membrane-bound and soluble proteins from this protective microenvironment—such as stromal cell-derived factor 1, B-cell activating factor, A PRoliferation Inducing Ligand (APRIL),4,6 vascular endothelial growth factor,7 and CD40 ligand (CD40L)8,9—increase resistance to spontaneous and drug-induced apoptosis, but much less is known about the mechanisms of proliferation of CLL cells.

B-cell receptor (BCR) signaling is believed to have a central role in CLL by promoting cell survival and proliferation and modulating migration and tissue homing.10 It has been suggested that, in an appropriate microenvironment, the BCR becomes engaged by microbial or autoantigens, which along with other costimulatory signals promote the expansion of the CLL clone.7 Also, very recently, cell-autonomous antigen-independent BCR signaling was reported as a crucial determinant in CLL.11 BCRs cloned from CLL samples but not from other B-cell malignancies induced autonomous Ca2+ signals, yet in vitro CLL cells do not proliferate or respond similarly to BCR ligation. In general, BCR ligation in immunoglobulin (Ig) H chain (IgH)-unmutated CLL leads to activating/proliferative responses, whereas it favors anergic/antiapoptotic responses in IgH-mutated CLL.12 This suggests that in addition to BCR-mediated mechanisms, other signals may also contribute to proliferation of CLL cells.

In proliferation centers, CLL cells are in close contact with activated CD4+ T cells,13 and it has been proposed that these cells can support the growth of CLL cells through CD40 ligation.14 In addition, 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.15 However, although CD40L stimulation alters the apoptotic profile of CLL cells and increases their resistance to apoptosis,8 it induces minimal proliferation on its own.10 Thus, there might be other stimuli provided by activated CD4+ T cells that contribute to proliferation of CLL cells.

Previously, a variety of T-cell-derived cytokines have been studied for involvement in the induction of proliferation in CLL cells.17,18 A prominent candidate is interleukin (IL)–21, a cytokine that has a fundamental role in the development of T-cell-dependent
B-cell responses has also been studied recently in the context of CLL proliferation. 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 natural killer T cells. Depending on the interplay with costimulatory signals and on the developmental stage of a B cell, IL-21 induces proliferation, differentiation into Ig-producing plasma cells, or apoptosis in both mice and humans. In CLL cells, direct stimulation with IL-21, or subsequent to CD40 stimulation, led to apoptosis. However, the presence of T cells in close proximity to CLL cells in proliferation centers led us to hypothesize that in this microenvironment, CLL cells could be exposed simultaneously to CD40L and IL-21 and that this could contribute to proliferation. Therefore, we investigated BCR-independent, T-cell-induced proliferation of CLL cells in vitro and the presence of IL-21 in LN from CLL patients.

Methods

Patient samples

Patient material was obtained from CLL patients, after informed consent, during routine follow-up or diagnostic procedures in our institute. The studies were approved by our Ethical Review Board and conducted in agreement with the Helsinki Declaration of 1975, revised in 1983. PB mononuclear cells (PBMCs) of CLL patients and healthy donors, 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, San Jose, CA) 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 were cultured as described.

Culture of CLL cells with autologous activated T cells

PBMC from CLL patients (>90% CD5+CD19+) cells were thawed, and after positive selection with CD19+ magnetic beads (MACS, Miltenyi Biotec B.V., Leiden, The Netherlands), CLL cells were labeled with 5 μM dodecylmethylammonium oxide-succinimidyl ester (DDAO-SE) (Invitrogen, Carlsbad, CA) and cocultured with autologous T cells (eluted fraction from CD19+ magnetic beads), activated with anti-CD3 (1 μg/mL, clone 1XE, Sanquin, Amsterdam, The Netherlands) and anti-CD28 (3 μg/mL, clone 15E8, Sanquin). Nonactivated (resting) T cells served as negative controls. To block CD40 and IL-21 signaling, anti-CD40L mAb (5 μg/mL, Abcam, Cambridge, UK) and anti-IL21RFc (20 ng/mL, R&D Systems, Minneapolis, MN) were used. As a control, the Fc portion of human IgG1 was used (20 μg/mL, R&D Systems). CLL cells were permeabilized (FoxP3 staining buffers, eBioscience, San Diego, CA) and stained with an antibody against Ki-67 (BD Biosciences).

Assessment of proliferation and apoptosis

PBMC (1.0 × 10^7 per milliliter) were labeled with 0.5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Life Technologies, Breda, The Netherlands) using the following primers: IL-2 forward 5′-GGCAACATGGAGAGGATTG-3′, IL-2 reverse 5′-GGCAACATGGAGAGGATTG-3′ and IL-21 forward 5′-GGCAACATGGAGAGGATTG-3′ and IL-21 reverse 5′-GGCAACATGGAGAGGATTG-3′. The results were normalized to 18S using the ΔΔt method.

Immunohistochemistry

Paraffin-embedded LN samples from CLL patients were cut, deparaffinized, and rehydrated through xylene and ethanol. Endogenous peroxidase was blocked by incubation in 0.03% H2O2 in methanol, for 20 minutes at room temperature, and then heat-induced epitope retrieval was performed in Tris-EDTA (pH = 9.0) buffer. Samples were incubated with anti-human IL-21 antibody (rabbit polyclonal, eBioscience) overnight at 4°C. Immunoreactive cells were visualized using BrightVision poly-HRP-anti-mouse/rabbit/tat IgG and BrightDAB (Immunologic, Duiven, The Netherlands), and counterstained with hematoxylin. Isotype control sections were prepared using normal rabbit IgG control antibody (Dako, Eindhoven, The Netherlands).

Statistical analysis

Differences between groups were compared using either the Student t test or the Mann-Whitney U test (paired, when applicable). P values < .05 were considered statistically significant.

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Results

Autologous activated T cells induce a gene profile highly similar to that induced by CD40L

We previously showed that prolonged CD40 stimulation of CLL cells mimics the situation in LN in terms of antiapoptotic profile and drug resistance. However, this model oversimplifies the complex interactions between T cells and CLL cells in the lymphoid organs and induces no or minimal proliferation. Therefore, we investigated more directly the signals provided by autologous activated T cells (Tact) to CLL cells.

We compared the gene expression profile induced by Tact and by CD40L stimulation alone. CD19+ cells from PB of CLL patients were cultured with purified, activated autologous T cells in T cell:B cell ratio of 1:2. Because we were interested in early response genes, gene expression was analyzed at t = 16 hours with whole genome microarrays (Gene Expression Omnibus accession number GSE50572). After normalization, unsupervised clustering was performed by principal component analysis. The first principal component (PC) is the mathematical combination of measurements...
Table 1. Gene sets upregulated in PLL by Tact or CD40L stimulation in comparison with PB

<table>
<thead>
<tr>
<th>Gene set description</th>
<th>Number of enriched gene sets*</th>
<th>Genes involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteasome</td>
<td>2</td>
<td>BIOCARTA_PROTEASOME_PATHWAY, KEGG_PROTEASOME, PSMA1, PSMA2, PSMA4, PSMA5, PSMD14, PSMD4, PSME2</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>2</td>
<td>KEGG_APOPTOSIS, REACTOME_APOPTOSIS, BCL2L1, BID, BIRC3, FAS, IL1B</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>5</td>
<td>REACTOME_CELL_CYCLE_MITOLOTIC, REACTOME_G1_S_TRANSITION, REACTOME_MITOLOTIC_M_M_G1_PHASES, REACTOME_SYNTHESIS_OF_DNA, DYNCl12, NDE1, NUP133, PCNA, PSMA1, PSMA2, PSMA4, PSMA5, PSMD14, PSMD4, PSME2, TUBG1, YWHAG</td>
</tr>
<tr>
<td>Cytokine–cytokine receptor interaction</td>
<td>1</td>
<td>KEGG_Cytokine_Cytokine_receptor_interaction, REACTOME_Cytokine_Cytokine_Receptor_interaction, CCL20, CCR5, CXCL2, FAS, IL1B, IL7, IL8</td>
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*Curated gene sets, canonical pathways. Only gene sets where at least 5 genes accounted for the enrichment score (leading edge genes) are included. Gene sets specific to nonlymphoid cell types are not shown.

that accounts for the largest amount of variability. Plotting PC1 vs PC2 distinguished the 3 different treatments (Figure 1A), and plotting PC1 vs PC3 showed a clear separation between PB and samples that were stimulated with either Tact or CD40L but not between the 2 in vitro systems. Next, we evaluated differential gene expression among the 3 groups. One hundred fifty-three genes were differentially expressed between any pair of groups (analysis of variance [ANOVA], P < .01, Bonferroni correction, minimum present calls = 1; Figure 1B and supplemental Table 2 [available on the Blood Web site]). As inferred from the previous analyses, 74% of these genes (113 out of 153) were modulated by both in vitro systems compared with PB, and only a minority of genes were different in 1 in vitro system alone (Figure 1C and supplemental Table 2). Gene set enrichment analysis was performed to interrogate which biological pathways were induced in PLL by Tact (Table 1). Both Tact and CD40L signaling regulated gene sets related to the proteasome, apoptosis, and cell cycle. Additionally, Tact differentially regulated a gene set related to cytokine-cytokine receptor interaction.

The induction of apoptosis-related genes was further validated by MLPA.29 Increased expression of BCL2L1 (Bcl-xL), BID, Fas (CD95), BIRC2 (cIAP1), BCL2A1 (Bfl-1), CDKN1A (p21), and SERPINB9 (P19), both by CD40L and Tact, was found (Figure 1D), albeit the levels after CD40L stimulation were in most cases higher. Induction of CD95 protein was also confirmed by FACS analysis (Figure 1E). These results suggest that the overall gene expression profile induced by Tact and CD40L stimulation in PLL cells is highly similar.

IL-21, in combination with CD40 stimulation, and Tact induce proliferation of PLL cells

The microarray analyses indicated that Tact induced cell cycle as well cytokine signaling gene sets. Using a similar system as described before with CD40 and CpG triggering,16,32 we therefore evaluated which cytokines in combination with CD40 stimulation could induce proliferation in PLL cells. Of note, stimulation with CD40 + IL-21 induced the highest levels of proliferation, in comparison with CD40 + anti-IgM, or CD40 + IL-15 (Figure 2A). As reported before, IL-2 did not induce proliferation in combination with CpG32 but not in combination with CD40 stimulation (Figure 2A-B). As expected, in normal B cells, anti-IgM + CD40 triggering did lead to proliferation (data not shown).

In combination with CD40L, IL-21 induced proliferation of PLL cells over a range of concentrations (Figure 2C). As previously reported,16 a small number of PLL samples proliferated somewhat in response to CD40L, but this was always increased both by IL-21 or a cytokine guanine dinucleotide (CpG) stimulation (Figure 2D), but not noticeably by IL-4, another Thelper cytokine (data not shown). The division index—the average number of cell divisions of a cell in the original population—was lower than 2 for all treatments (Figure 2E). To check whether this was due to shortage of nutrients/stimulation or a block in proliferation, we refreshed the stimuli and medium every 3 to 4 days, which led to proliferation of the majority of PLL cells (Figure 2F). Previous reports had suggested that IL-21 alone or subsequent to CD40 stimulation could induce apoptosis.26,27 In agreement, we found that in absence of CD40, IL-21 induced significant levels of apoptosis (Figure 2G), but this was largely diminished by the CD40 + IL-21 combination. Thus, in the presence of CD40L stimulation, IL-21 signaling results in proliferation of PLL cells, with a significant decrease in the induction of cell death.

We next investigated changes underlying the switch from apoptosis to proliferation upon CD40 + IL-21 stimulation. Freshly thawed PLL PBMCs were incubated overnight on CD40L-expressing cells in the presence or absence of IL-21. Microarray analysis revealed that 51 genes were significantly regulated by IL-21 in CD40L-stimulated cells (P < .01, fold change >3; Figure 3A and supplemental Table 3). Of these, 17 genes were upregulated and 34 downregulated, among which we found a significant decrease in BCL2L1 (Bcl-xL) (Figure 3A). To validate this, gene expression of Bcl-xL and other apoptosis-related mediators in PLL cells stimulated with CD40L and/or IL-21 were analyzed via MLPA assay. As described previously,8,28 in vitro CD40 stimulation of PLL cells induced the transcription of BCL2L1 (Bcl-xL) and BCL2A1 (Bfl-1) and led to a decrease in the expression of BCL2L1 (Bim) (Figure 3B). IL-21 interfered with Bcl-xL upregulation induced by CD40L but did not affect Bim downregulation, both at RNA and protein levels (Figure 3B-C). In addition, Mcl-1 protein was also upregulated by CD40 + IL-21, in comparison with CD40 alone, compensating for the increase in Noxa protein (Figure 3C). Overall, the balance in pro- and antiapoptotic Bcl-2 members appeared in line with the observed shift to a prosurvival state of the CD40 + IL-21 combination, in comparison with IL-21 alone.

IL-21 is known to engage Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling in PLL.21 In agreement, STAT3 expression and phosphorylation were induced as detected by microarray and western blotting (Figure 3A-D). IL-21 caused sustained STAT3 but not STAT5 activation (data not shown). To establish the requirement for JAK signaling, we added a pan-JAK inhibitor to the system, and this indeed prevented proliferation (Figure 3E-F).
In accordance with previous studies in healthy B cells and CLL cells, we detected a significant induction of granzyme B by IL-21 in CD40L-stimulated CLL cells. Granzyme B induction after IL-21 was confirmed by flow cytometry and was more marked after CD40 stimulation (supplemental Figure 1B). Of note, the increase in Granzyme B was not accompanied by expression of perforin, and IL-21 treatment did not alter the percentages of Granzyme B<sup>+</sup> or Granzyme B<sup>+</sup> Perforin<sup>+</sup> CD3<sup>+</sup> cells in the same cultures (supplemental Figure 1B).

To investigate whether the Tact system could also induce proliferation in CLL cells, we assessed Ki-67 staining after 2 days of stimulation. Clearly, Tact induced a fraction of CLL cells to become positive for Ki-67 (Figure 4A). Importantly, Ki-67 staining of CLL cells was reduced in the presence of a blocking antibody for CD40L and a decoy receptor for IL-21. This blocking effect of anti-CD40L and IL-21-Fc was not always complete but appeared specific, as demonstrated by the control IgG1 Fc molecule (Figure 4A-B).

**IL-21 + CD40 gene signature is induced in CLL cells by activated T cells and is also present in lymph node samples**

We compared gene expression profiles of IL-21 + CD40 stimulation with Tact. From the averaged fold induction of all genes modulated by IL-21 in comparison with CD40L-stimulated cells, we compiled an “IL-21 score.” This score was 5 times higher in CLL cells after stimulation with CD40L + IL-21 (Figure 5A). The same score was calculated in the microarray dataset from CLL cells cocultured with autologous Tact, and also in this case the IL-21 score was significantly higher than in matching CD40L-stimulated cells (Figure 5B). Next, we investigated whether the expression signature as summarized in the IL-21 score was present in LN and BM samples from CLL.
patients. We calculated the score from 5 BM samples and 2 LN samples in comparison with their matched PB samples. In the LN samples, the values obtained (1.5 and 1.6) were higher than those found in matching BM samples. This was suggestive of IL-21 biological activity but inconclusive because of the low number of LN samples available to us (data not shown). To investigate this issue further, we interrogated an available dataset generated by Herishanu et al, who investigated matched PB, BM, and LN samples using the same microarray platform. The IL-21 scores generated from their public deposit are depicted in Figure 5C. Most of the LN samples in this external collection presented IL-21 scores significantly higher than those of the paired BM samples, strengthening the validity of the IL-21 score. Collectively, the data are indicative of active IL-21 signaling in CLL in vivo and suggest that the IL-21 signature is higher in the LN than in the BM of CLL patients.

**Lymph node samples from CLL patients contain IL-21, which can be produced in vitro by Tfh cells**

Finally, we explored whether we could directly detect IL-21 RNA and protein in LN samples from CLL patients. We measured IL-21 by quantitative reverse-transcription polymerase chain reaction (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 in healthy and CLL samples, in comparison with the control condition. A similar analysis was performed on frozen LN samples of CLL patients, where we could also detect IL-21 mRNA, albeit at variable levels (Figure 6A). In order to confirm these results, we performed immunohistochemistry on paraffin-embedded LN samples. Staining conditions were optimized using a reactive lymph node from a patient with systemic lupus erythematosus (supplemental Figure 2) and then applied to 5 available CLL LN samples. Of these, 3 showed clear IL-21 positive cells (2 shown in Figure 6B). Together, these data strongly suggest the presence of IL-21 in at least some CLL LN tissues.

To address which population of CD4+ T cells from LNs from CLL patients were able to produce IL-21, we stimulated matched samples of PB and LN from 2 CLL patients with PMA and ionomycin for 4 hours. Because the main producers of IL-21 are follicular helper T cells (Tfh), characterized by CXCR5 expression, we evaluated CD40L expression and IL-21 production in CXCR5+ vs CXCR5− CD4+ T cells. The PB percentage of CXCR5+ cells was comparable to what we have observed in healthy LNs in comparison with matched PB (Simone H.C. Havenith, Ester B.M. Remmerswaal, Mirza M. Idu, Karlijn A.M.I.)
van Donselaar-van der Pant, Nelly van der Bom, Frédérique J. Bemelman, Ester M.M. van Leeuwen, Ineke J. M. ten Berge, and René A. W. van Lier, manuscript submitted July 2013), one sample in particular presented much higher levels of CXCR5$^+$ cells in the LN in comparison with PB (Figure 6C). Most important, for both patients and compartments, the highest production of IL-21 was found in CXCR5$^+$ CD40L-expressing cells. These results suggest that activated T cells with a Tfh phenotype are an important source of IL-21 in CLL patients.

Discussion

The mechanisms of CLL proliferation and its pharmacological targeting are of obvious clinical interest. We describe for the first time that autologous, activated T cells induce proliferation of CLL cells. The combination of CD40L and IL-21 stimulation alone is sufficient to induce robust proliferation of CLL cells. This aspect was recently also reported independently by Ahearne et al.23 The gene signature derived from combined CD40 + IL-21 stimulation could be traced in in vivo lymph node samples where CD4$^+$ T and CLL B cells have been proposed to interact.3,14 On the basis of our combined molecular and cellular analyses, we suggest that IL-21 may play a role in antigen-independent proliferation of CLL cells at lymph node sites.

In the CD40 + IL-21 and the Tact system, the CLL cells start to proliferate after 2 to 3 days. Because an early time point of 16 hours after stimulation was chosen for RNA sampling, no drastic changes in genes expression related to cell cycle are expected yet and indeed were not observed. A previous study on gene expressions pattern induced in CLL by CD40 stimulation applied later time points (48-72 hours)40 and noted that in comparison with healthy donor B cells, CLL cells displayed reduced expression of cell cycle genes.

Several previous reports on the effect of IL-21 on CLL have highlighted apoptosis induction by IL-21 alone on freshly isolated CLL cells.26 In another set-up, CLL cells were first stimulated with CD40L before addition of IL-21.27 It has been shown for murine B cells that IL-21 can induce apoptosis if administered together with lipopolysaccharide or CpG but promotes proliferation when combined with anti-IgM or anti-CD40 agonist antibodies.25 In our experiments, sole IL-21 also induced apoptosis, but concomitant CD40-signaling clearly shifted the balance to proliferation. Our results suggest that although clinical application of IL-21 could potentially eliminate some CLL cells, it poses the risk of inducing proliferation of CLL cells in contact with activated T cells.

An important point of the present work was to find evidence of IL-21 presence/signaling in vivo. First, we used an indirect strategy, which has also been used to detect BCR signaling in LN from CLL patients.36 An IL-21 score was computed as the average of the fold change in messenger RNA expression of the 51 genes shown in Figure 3A and in supplemental Table 3. (A) IL-21 score for CD40 versus CD40 + IL-21 for samples 2, 5B, 11, and 30. (B) IL-21 score for CD40 versus Tact, for samples 2, 5B, 19, 24, and 27. (C) IL-21 score calculated from published data of Herishanu et al.36 from BM and PB, in relation to the corresponding level in PB.
Figure 6. IL-21 expression in lymph nodes from CLL patients. (A) IL-21 expression was studied by qRT-PCR in RNA from PBMCs from 2 healthy donors and 2 CLL patients (samples 6A, 8), after activation with CD3 or CD28 antibodies or PMA + ionomycin, or in total frozen LN samples from CLL patients (samples 34 to 40). (B) IL-21 expression was analyzed in 2 paraffin-embedded CLL LN samples by IHC. Upper panels show nonspecific staining with control antibodies; lower panels show results with anti-IL-21 antibodies. Original magnification ×40, further digital magnification ×4. (C) Matched samples of PB and LN from 2 CLL patients (samples 11 and 14) were stimulated as indicated in the "Methods" section for 4 hours. They were then stained for expression of CD3, CD4, CXCR5, CD40L, and IL-21. Results depict the histograms for CXCR5 expression (unstimulated samples, left panels) and the expression of IL-21 and CD40L by CXCR5+ and CXCR5− CD3+ CD4+ cells for PB (upper) and LN (lower) samples.
to that achieved by the combination of IL-21 and CD40L-expressing cells. More important, both in samples from our cohort and in data available from a previous publication,\textsuperscript{36} we found a small but consistent increase in these scores in comparison with those found in BM samples. The low scores are to be expected because they were obtained from the total CLL B-cell population of these organs, and the amount of CLL cells potentially in contact with IL-21 could be small, in contrast to the in vitro systems. To estimate the size of this subpopulation, one approximation is to consider the population of proliferating cells as potentially influenced by the cytokine. Herishanu et al reported that Ki-67\textsuperscript{+} cells represent between 3% and 8% of the CD3\textsuperscript{+} cells in the LN.\textsuperscript{36} Given these modest percentages, it seems therefore of relevance that we could in fact find significant differences in the IL-21 score in LN samples. A second method was to directly demonstrate the presence of IL-21 in LN samples by qRT-PCR and immunohistochemistry (IHC). The qRT-PCR data indicate that the levels of IL-21 in LN samples were in the range of T cells activated in vitro by anti-CD3 antibodies, which given the point addressed above about cell percentages, seems significant. The IHC data were not conclusive for all samples studied but were strongly suggestive of pericellular and cytoplasmic presence of IL-21 in certain areas. More important, an important source of IL-21 after in vitro activation of PB and LN were the CXCR5\textsuperscript{+} CD4\textsuperscript{+} T cells. This holds interesting implications, especially in view of the recently reported increased levels of CXCR5\textsuperscript{+} Th1-like cells in PB from CLL patients.\textsuperscript{23} Because Th1 differentiation and maintenance has been described to be dependent on the interaction with B cells,\textsuperscript{41} it might well be that the interaction of T cells with CLL cells leads to this increase in Th1-like cells.

It has recently become evident that high levels of proliferation of the leukemic population in CLL are correlated with worse prognosis.\textsuperscript{32} The circulating Ki-67 index in plasma significantly correlated with lymph node involvement and high values of this index were associated with shorter survival.\textsuperscript{43} The recently reported antigen-independent propensity for autonomous BCR signaling might be a crucial contribution to proliferation in CLL.\textsuperscript{10} Of note, although BCRs cloned from CLL samples generated clear autonomous Ca\textsuperscript{2+} signals, these were well below the levels observed upon BCR triggering. Because it is well known that CLL cells cannot proliferate without external stimuli in vitro, it is not likely that low-level autonomous Ca\textsuperscript{2+} fluxes are themselves sufficient to drive proliferation in vivo. Therefore, additional mechanisms, possibly involving T-cell-mediated CD40 and IL-21 signaling, might play a supplemental role.

In this work, we have shown that stimulation with Tact and in particular CD40 + IL-21 is sufficient to induce antigen-independent proliferation of CLL cells. We also found strong indications for 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.

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Authorship
Contribution: M.F.P. designed and performed research, interpreted data, and wrote the manuscript; M.J. and J.M.T. designed and performed research and collected and interpreted data; E.B.M.R., R.T., M.H.A.V.A., G.G.v.B., and I.A.M.D. designed and performed experiments; D.M.L. collected patient samples and information; M.H.J.v.O., S.T.P., R.A.W.v.L., and A.P.K. designed and supervised experiments and interpreted data; and E.E. designed and supervised experiments, interpreted data, and wrote the manuscript.

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