The microenvironment and treatment resistance in chronic lymphocytic leukemia

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Activated T cells induce activation, proliferation and drug resistance in chronic lymphocytic leukemia cells

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Abstract

CLL is a dynamic disease with substantial proliferation rates in vivo. CLL cells rapidly undergo apoptosis in vitro indicating the important role of signals from the microenvironment such as bone marrow and secondary lymphoid organs. To study the mechanisms of proliferation and drug resistance of CLL cells, robust and reliable experimental systems must be developed that resemble the lymph node microenvironment. An important signal in the lymph node microenvironment is CD40L activation of CLL cells by activated T cells. Here, two in vitro systems, mimicking CLL T cell interactions, were compared; a CD40L transfectant culture system and a system that depends on the coculture of CLL cells with autologous activated T cells. Both in vitro systems were highly comparable as to activation and drug resistance of CLL cells. Activated autologous T cells however also induced proliferation, thereby most closely mimicking the in vivo situation. Proliferation of CLL cells was dependent on simultaneous activation with CD40L and IL-21, making these molecules possible targets in the treatment of CLL. Interestingly, gene expression profiles of CLL cells co-cultured with either CD40L transfectants or activated autologous T cells resembled transcriptomes of CLL cells obtained from freshly isolated lymph nodes. We conclude that signals derived from T cells are instrumental in inducing and maintaining activation, drug resistance and proliferation of CLL cells.
Introduction

Despite major advancements in treatments, chronic lymphocytic leukemia (CLL) remains an incurable disease. CLL cells were considered to be long-lived tumor cells arrested in the G0/G1 phase of the cell cycle and possessing intrinsic apoptosis defects, a concept largely based on analyses of peripheral blood derived CLL cells. However, studies of in vivo cellular kinetics, strongly indicated that CLL is a dynamic disease with substantial proliferation rates as well as increased death rates compared with normal B cells (1, 2). CLL cells relentlessly accumulate in vivo, but rapidly undergo spontaneous apoptosis in vitro, suggesting their apoptosis resistance in vivo depends on external signals. Indeed, growing evidence suggests that survival signals from the microenvironment (bone marrow and secondary lymphoid organs) not only promote disease progression but also result in an inability to eliminate the leukemic clone (reviewed in (3)).

Several in vitro models have been used to mimic the natural microenvironment. CLL cells co-cultured with nurselike cells (NLCs) (4), bone marrow stromal cells (MSCs) (5) or follicular dendritic cells (6) are protected against apoptosis. In CLL tissues pseudofollicular structures are found (3, 7) consisting of CLL cells, antigen (Ag)-presenting cells and numerous CD4+ T cells (8, 9). It has been shown that CLL cells in lymph nodes (LNs) attract CD4+ T cells (8) by producing CCL4 and CCL22 (10, 11), and that these T cells express CD154 (CD40L) (9). In vitro stimulation of CLL cells with CD40L results in an increased anti-apoptotic profile (12-14) and rescues them from drug-induced apoptosis (12, 13, 15). Based on these observations CD40L stimulation in vitro is considered to mimic the lymph node microenvironment in CLL. However, this model might be oversimplified, since it reflects only the CD40L-CD40 interaction and not other cell-related membrane-bound or soluble factors. Therefore, the important question on how to construct a model which most faithfully reflects the in vivo CLL LN microenvironment remains to be answered.

To dissect the role of T cells in activation, proliferation and drug resistance we designed a new in vitro CLL LN system by co-culturing CLL cells with activated autologous T cells. As control we cultured CLL cells on CD40L-expressing mouse fibroblasts (3T40L) as described previously (12). In both in vitro systems, we studied activation, proliferation and drug resistance of CLL cells and compared gene expression profiling.

Methods

Patient samples

Patient material (peripheral blood, bone marrow aspirate) was obtained from CLL patients (diagnosed according to the NCI-WG guidelines) after routine follow-up or diagnostic procedures at the department of hematology of the Academic Medical Center Amsterdam. Peripheral blood mononuclear cells (PBMCs) and bone-marrow derived mononuclear cells were isolated by Ficoll density gradient centrifugation.
(Pharmacia Biotech, Roosendaal, the Netherlands) and either used immediately or stored in liquid nitrogen. Lymph node material diffusely infiltrated by B-CLL cells (>95% lymphocytes) was freshly frozen in liquid nitrogen directly after surgical removal and after thawing LN-derived CLL cells were isolated by mincing the lymph nodes. During all in vitro experiments, cells were maintained in culture medium: Iscove’s modified Dulbecco medium (IMDM: Gibco Life technology, Paisley, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100μg/ml gentamycin and 0.00036% β-mercaptoethanol. All 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 written informed consent was obtained in accordance with the Helsinki Declaration of 1975, revised in 1983.

Reagents
The proteasome inhibitor bortezomib was obtained from Janssen-Cilag (Tilburg, The Netherlands). Fludarabine (F-Ara-A) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Cell cultures
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 B cells were labelled with 5 μM DDAO according to the manufacturer’s recommendations (Invitrogen, Breda, The Netherlands). After labelling with DDAO, CLL B cells were either stimulated with CD40 ligand (CD40L) transfected NIH3T3 (3T40L) cells as described previously(12) or co-cultured with autologous T cells (negatively selected with CD19+ magnetic beads (MACS, Miltenyi Biotec B.V. Leiden, The Netherlands) activated with CD3 (clone 1XE, Sanquin, Amsterdam, The Netherlands) and CD28 (clone 15E8, Sanquin, Amsterdam, The Netherlands). Non-transfected 3T3 cells or non-activated (resting) T cells respectively were used as negative controls. In parallel experiments, split well cultures were performed; CLL cells were seeded onto transwell (TW) diffusion chambers (0,4 μm microporous filter, Falcon, Beckton Dickinson, Heidelberg, Germany) and placed into 3T40L-coated or activated T cell seeded 6 well-plates. To block CD40 signaling and IL-21 signaling, anti-CD40L mAb (5μg/ml, Abcam, Cambridge, UK) and anti-IL21R mAb (10μg/ml, R&D systems, Minneapolis, USA) were used respectively. After 2 days, CLL cells were gently removed from the fibroblast layer or resuspended in cultures with activated T cells and TW inserts and used in further experiments.

Proliferation and activation assays, induction and analysis of apoptosis
To analyze proliferation upon CD40L with and without IL-21 stimulation, CLL cells were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes). Cells were resuspended in PBS at 1,0 x 10^7/mL in 0,5 μM CFSE for 15 min at 37°C and washed in IMDM containing 10% FCS. After labelling, cells were cultured
on 3T40L cells in the presence or absence of 25 ng/ml IL-21 (16). After 5 days, cells were analyzed by FACS for CFSE staining. For intracellular Ki-67 expression analysis, cells were fixed and permeabilised (eBioscience, San Diego, CA) and subsequently stained with FITC-conjugated Ki-67 or isotype control (Becton Dickinson, San Jose, CA). To determine expression of the activation markers CD95 and TNFR2 cells were stained with mAb conjugates APC-conjugated CD5, PerCP-conjugated CD19 and FITC-conjugated CD95 (all Beckton Dickinson, San Jose, CA) or PE-conjugated TNFR2 (Lot # LFB 046021, R&D systems Minneapolis, USA). For apoptosis induction, cells were treated with 10μM fludarabine (48 hrs) or 7.5nM bortezomib (24 hrs). Apoptosis was analyzed by evaluation of mitochondrial membrane potential with DioC6 (Molecular probes, Leiden, The Netherlands) according to the manufacturer’s recommendations. Expression of intracellular protein, cell surface molecules and DioC6 was determined using the FACSCalibur flow cytometer (BD biosciences) and CellQuest software (Beckton Dickinson) was used for data acquisition. Data were analyzed with FlowJo software (TreeStar, San Carlos, CA, USA).

Sample preparation for microarray analysis
Matched peripheral blood (PB), bone marrow (BM), and lymph node (LN) samples were collected from treatment-naïve CLL patients (Table 1). Mononuclear cells were isolated by Ficoll density gradient centrifugation (Pharmacia Biotech, Roosendaal, the Netherlands) and stored in liquid nitrogen until use. For stimulation with 3T40L cells or autologous activated T cells, mononuclear cells were thawed and CLL cells were positively- and T cells negatively selected with CD19+ magnetic beads (MACS, Miltenyi Biotec B.V. Leiden, The Netherlands). After selection the percentage CD5+CD20+ CLL cells and CD3+ T cells was 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). In parallel experiments CLL cells were cultured on 3T40L cells. Cells were stimulated ON at 37°C in a 5% CO2 incubator or left unstimulated at 4°C. Mononuclear cells from LN and BM were thawed together with the PBMCs and kept at 4°C 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, San Jose, CA). CD5+CD19+ or CD5+CD20+ cells were sorted in a FACS Aria (BD Biosciences, San Jose, CA). Purity was > 98% in all cases. Immediately after sorting, cells were washed with PBS, resuspended in Trizol (Invitrogen, Leiden, The Netherlands) and stored at -80°C. RNA was extracted according to the Trizol’s manufacturer instructions and then cleaned with RNeasy kits (Qiagen, Venlo, The Netherlands) to obtain high purity RNA. The concentration and purity of the RNA were determined with a NanoDrop spectrophotometer ND-1000 (NanoDrop Technologies,
Wilimington, USA) and the ribosomal RNA band integrity was determined on an Agilent 2001 BioAnalyzer® (Agilent Technologies, Santa Clara CA, USA).

Microarray data analysis
Fragmentation of cRNA, hybridization to HG-U133 Plus 2.0 microarrays (Affymetrix, Santa Clara CA, USA) and scanning was carried out according to the manufacturer’s protocol (Affymetrix) 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 MAS5.0 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 affymetrix mas5 algorithm, normalized to a trimmed mean of 100. In-built differential gene expression as well as KEGG PathwayFinder algorithms were applied between groups.

Results

Stimulated T cells induce activation of CLL cells in both CD40L dependent and independent ways
To assess the role of T cells in activation of CLL cells, DDAO-labelled CLL cells were cocultured with CD3-CD28 activated autologous T cells. Patient characteristics are listed in Table 1. The CLL-T cell coculture system was compared with the earlier described

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Patient characteristics including sex, age, mutation of IgHV genes, genetic aberrations, p53 function, Rai stage and previous therapy. (F = female, M = male, Mut = mutated IgHV genes, UM = unmutated IgHV genes, ND = not determined)
CD40L transfectant system (12, 17, 18). Non-activated (resting) T cells or non-transfected 3T3 cells respectively were used as negative controls. A blocking CD40L mAb was used to inhibit CD40-CD40L interaction. In the presence of a CD40 mAb (to prevent internalization of CD40L after ligation with its ligand (19)), activated T cells from CLL patients showed marked increase of CD40L (Figure 1A). CLL cells cocultured with activated T cells showed increased blast-like appearance comparable with CLL cells cultured on CD40L transfectants (Figure 1B, gated on DDAO+ cells). A decrease in percentage blast-like cells was observed in the presence of anti-CD40L mAb, indicating a role for CD40L in inducing activation of CLL cells in both systems.

A well-known activation marker is CD95 or Fas. It has been shown that CLL cells upregulate CD95 after CD40 stimulation \textit{in vitro} (12) and \textit{in vivo} (20). Activated T cells also induced CD95 upregulation in CLL cells but remarkably this was independent of CD40L (Figure 1C,D). To test whether soluble factors produced by activated T cells affected the CLL cells, split-well experiments were performed. In these experiments CD95 upregulation was observed in CLL cells cultured with activated T cells, but not with CD40L transfectants. A similar pattern was observed for the upregulation of another TNFR-family member: TNFR2 (Figure 1D).

In summary, these data show that both CD40L and soluble factors produced by activated T cells regulate the activation of CLL cells and that the induction of CD95 can occur independent of CD40L. As described earlier for the CD40L system (12), increased CD95 expression on CLL cells did not result in increased sensitivity to FasL-mediated cell death (data not shown).

Activated T cells induce drug resistance in CLL cells via a CD40L independent mechanism

CD40 stimulation results in an increased anti-apoptotic profile (12-14) and drug resistance in CLL cells (12, 13, 15). To test whether T cells could also induce drug resistance, DDAO labelled CLL cells cocultured 48 hours with CD3-CD28 activated T cells were incubated with different cytostatic drugs (Fludarabine and Bortezomib). Apoptosis was analyzed by measuring DioC6 signal in DDAO+CD19+ CLL cells (for gating strategy see Figure 2A). Figure 2B shows significant drug resistance in CLL cells cultured with activated T cells. In contrast to CD40L transfectant-induced drug resistance, T cell-induced drug resistance was not blocked in the presence of anti-CD40L mAb. Interestingly, also in the absence of cell-cell contact, activated T cells induced drug resistance in CLL cells (Figure 2B, Tact in split well). Altogether the data indicate that activated T cell induced drug resistance in CLL cells is CD40L-independent and may depend on a soluble factor.
Gene expression profiling reveals strong similarities between CD40L and activated T cell stimulated CLL cells

To compare gene expression profiles of CD40-stimulated CLL cells and CLL cells cocultured with activated autologous T cells, microarray studies using Affymetrix technology were performed. For comparison CLL cells from peripheral blood (PB) were used. Gene expression profiles of CLL cells from both in vitro systems (CD40L transfectant system and activated T cell system) were very similar (Figure 3), with the exception of 14 genes upregulated in CLL cells cocultured with T cells (data not shown). It should be noted that microarrays from CLL co-cultured with T cells showed CD3 delta, epsilon and gamma message indicating that despite rigorous cell sorting low amounts of activated T cells contaminated these samples (data not shown).

Altogether, these data show that the CD40L transfectant- and activated T cell system are comparable in terms of activation of CLL cells and upregulation of NF-κB targets likely leading to drug resistance. The NF-κB signature was also found in LN CLL samples. Further, both in vitro systems resemble in vivo arrays of lymph node CLL cells. However, because of residual T cells in the activated T cell system no firm conclusions can be
Figure 2. Autologous activated T cells induce drug resistance in CLL cells. DDAO labelled CLL cells cocultured 48 hours with CD3-CD28 activated T cells were incubated with different cytostatic drugs (Fludarabine 10 μM for 48 hours and Bortezomib 7.5 nM for 24 hours). Resting T cells were used as negative control and CD40L transfectant-induced drug resistance as positive control. Apoptosis was analyzed by evaluation of mitochondrial membrane potential with DioC6. A. Facs plots showing gating strategy for apoptosis analysis in CLL cells in different CLL-T cell co-culture systems. Apoptosis was analyzed by measuring DioC6 signal in DDAO+CD19+ CLL cells. Left: medium control, right: fludarabine treated cells. B. Bar graphs showing drug-induced CLL cell apoptosis in the CLL-T cell coculture system (left) and 3T3-CD40L transfectant system (right). Activated T cell-induced drug resistance is not abrogated in the presence of aCD40L mAb in contrast to 3T40-induced drug resistance. Averaged results from 11 CLL patients are presented as percentage apoptosis (mean ± SEM). 01<p< .05 *, p<.001 ***
drawn concerning putative differences in the expression of ubiquitous genes (e.g. cell cycle/proliferation genes) in both systems.

**Proliferation of CLL cells is both dependent on CD40L and IL-21.** Although gene expression profiles of both *in vitro* systems were similar, activated T cells can induce CD95 upregulation and drug resistance in CLL cells independently from CD40 signaling (Figure 2). These findings suggest that (*in vivo*) lower levels of CD40L cooperate with soluble factors to induce activation, drug resistance and possibly proliferation, an important characteristic of CLL cells *in vivo* (1, 2). Therefore the question arose which soluble factor(s) could have this role *in vivo*. Proliferation is not observed *in vitro* without the addition of cytokines and/or membrane-bound factors but, CLL cells from lymph nodes show higher percentage Ki-67+ cells than CLL cells from peripheral blood or bone marrow (Figure 5A). This finding underscores the importance of the microenvironment for CLL behaviour *in vivo*. The cytokine IL-21 has been known to play an important role in the homeostasis of normal B cells (25). It has been shown that IL-21 plays an essential role in T-cell induced B cell activation, proliferation, plasma cell (PC) differentiation and immunoglobulin (Ig) production (26). Furthermore, peripheral blood memory B cells can be converted into highly proliferating Ig-secreting B cells by introducing Bcl-6 and Bcl-XL and by culturing these cells with CD40 ligand (CD40L) and interleukin-21 (IL-21) (16). Therefore, it was tested whether culturing CLL cells with CD40L and IL-21 also resulted in proliferation. Cells were labelled with CFSE and after five days the number of divisions was analyzed by flow cytometry (Figure 5B). Clearly, CLL cells cultured on CD40L transfectants and in the presence of IL-21 showed significant proliferation in contrast to CLL cells cultured on CD40L transfectants only (Figure 5B,C). CD40L and IL-21 are two factors produced by follicular helper T cells (reviewed in (27)). Therefore, it was tested whether CLL cells cocultured with activated T cells also started to proliferate and if so, whether this was blocked by aCD40L- or aIL21R blocking mAb. Resting T cells were used as negative control and CD40L transfectants in combination with IL-21 were used as positive control. In CLL cells cocultured with activated T cells a significant increase in Ki-67+ cells was observed which could be blocked with IL-21R blocking mAb and with aCD40L blocking mAb (Figure 5D,E left). Interestingly, activated T cell induced upregulation of CD95 was also inhibited in the presence of IL21-R blocking mAb (data not shown). No proliferation was found in CLL cells cocultured with resting T cells, or cultured on 3T3 or CD40L transfectants alone. Again a large increase in Ki-67+ cells was observed in CLL cells cultured on CD40L transfectants in the presence of IL-21 (Figure 5D,E right). These data show that activated autologous T cells induce proliferation of CLL cells which is dependent on IL-21 and CD40L (27). In sharp contrast, CLL cells cultured on CD40L transfectants alone don’t proliferate. Interestingly, the IL-21R was upregulated in both *in vitro* systems when compared with peripheral blood CLL cells (Figure 5F).
Figure 3. Gene expression profiling in CD40L transfectant- and activated T cell system. Heatmap of 474 genes differentially expressed in peripheral blood CLL cells compared to peripheral blood CLL stimulated with CD40L or activated T cells for 24 hours. Shown are mean changes in transcript levels. Shades of red and green represent up- and downregulation of genes, respectively. The gene set included all genes that were significant after the Bonferroni correction for multiple testing ($P \leq 0.0001$). Samples were clustered together with mean expression value. The CD40L transfectant and activated T cell system induce major changes in gene expression compared with peripheral blood and similar gene expression was observed in both in vitro systems, with the exception of a set genes indicated by the white boxes.

Figure 4. Upregulation of NF-κB targets in CLL cells co-cultured with CD40L transfectants or activated T cells. Bar graphs showing gene expression of NF-κB targets Bcl-XL (A), Bfl-1 (B) and TNF (C) from CLL cells co-cultured with CD40L transfectants or activated T cells compared with peripheral blood samples. Data are presented as gene expression in arbitrary units (mean ± SEM).
Figure 5. Proliferation of CLL cells in lymph nodes is both dependent on CD40L and IL-21.

A. CD5+CD19+ CLL cells isolated from peripheral blood (PB), bone marrow (BM) and lymph nodes (LN) were analyzed for Ki-67 expression. Averaged results from 2 CLL patients are shown. B. CLL cells were labelled with CFSE and cultured on CD40L transfectants in the presence and absence of IL-21. After five days the number of divisions was analyzed by flow cytometry. Shown is a representative histogram of one of 10 patients tested. C. Bar graph showing the percentage proliferating CLL cells (positive CFSE dilution) cultured on CD40L transfectants in the presence and absence of 25 ng/ml IL-21. Averaged results from 10 CLL patients are presented as percentage proliferating cells (mean ± SEM). .001 < p < .01 **. D. CLL cells were labelled with DDAO and cocultured with activated T cells and after 48 hours. Facs analysis of Ki-67 expression in DDAO+ CLL cells was performed. Resting T cells or 3T3 cells were used as negative control and CD40L transfectants in combination with IL-21 was used as positive control. Experiments were done in the presence or absence of aCD40L- or aIL-21R blocking mAb. E. Left: CLL cells were co-cultured with activated T cells in the presence or absence of aCD40L-or aIL-21R blocking mAb. After 48 hours the percentage Ki-67+ CLL cells was analyzed by
flow cytometry. Data are presented as percentage Ki-67+ CLL cells relative to activated T cells (mean ± SEM of 8 patients tested). Right: CLL cells were cultured on CD40L transfectants in the presence and absence of IL-21 or αCD40L. After 48 hours the percentage Ki-67+ CLL cells was analyzed by flow cytometry. Bar graph presents averaged results from 9 CLL patients (mean ± SEM). 01 < p < 0.05 *, p < 0.001 **. F. IL-21R gene expression. CLL cells were stimulated with CD40L transfectants (left) or activated T cells (right). Peripheral blood CLL cells were used as control (middle). After sorting RNA was isolated and microarray analysis was performed using affymetrix as described in materials and methods. Gene expression of IL-21R was compared between groups.

Discussion

To investigate the role of the microenvironment in CLL, several in vitro systems have been described (3-6, 12). This study shows that for mimicking the lymph node microenvironment in vivo in CLL, two in vitro systems are robust and reliable; the CD40L transfectant system and a CLL-activated T cell coculture system which both induce activation and drug resistance of CLL cells. However, an important characteristic of in vivo CLL cells, proliferation (1, 2), is only induced by activated T cells, suggesting this system more faithfully reflects the in vivo CLL LN microenvironment. Interestingly, proliferation appeared to depend on IL-21, a cytokine mainly produced by follicular helper T cells in lymph nodes (27, 28). Finally, the microarray data show that the CD40L transfectant system and the CLL-activated T cell coculture system are highly similar, and both in vitro systems resemble the in vivo lymph node situation with respect to increased NF-κB activity.

Activation of CLL cells by allogeneic activated T cells was already shown by Ranheim et al (29). CLL cells cocultured with CD4+ mitomycin C-treated T cells previously plated in culture wells coated with α-CD3 mAb dramatically increased expression of CD54 (ICAM-1) and B7/BB1. Upregulation of CD54 (ICAM-1) and B7/BB1 was significantly inhibited in the presence of anti-CD40 mAbs and cell-cell contact was required. The authors suggested that CD40 crosslinking may be necessary and sufficient for T-cell-induced expression of B7/BB1 and other accessory molecules on CLL cells. In our study, autologous activated T cells induced activation of CLL cells, which was partly dependent on CD40L. The percentage of blast-like CLL cells in cocultures of activated T cells and CLL cells was decreased in the presence of anti-CD40L mAb, but upregulation of CD95 expression was not affected by anti-CD40L mAb. Furthermore, increased expression of CD95 was also observed in CLL cells cultured with activated T cells in a split well system. These data suggest that a soluble, non-membrane bound factor is sufficient for the upregulation of CD95. Differences in signaling strength could explain a variable dependence on cytokines between the various studies (29).

Another important characteristic of in vivo CLL cells is drug resistance induced by microenvironmental factors (3). It is of clinical importance to develop a reliable and stable in vitro system to study the underlying mechanisms of drug resistance in CLL. The CD40L transfectant system has been extensively used. CLL cells cultured on CD40L transfectants show an increased anti-apoptotic profile (12-14) and drug resistance to
several different cytostatic drugs (12, 13, 15). Tinhofer et al showed the pro-survival potential of autologous CD4+ memory T cells on B-CLL cells in vitro (30). Our study shows for the first time that autologous activated T cells are also capable of inducing drug resistance in CLL cells. In contrast to the CD40L transfectant system, activated T cell induced drug resistance was not solely dependent on CD40L. T/B cell contact was not required to induce drug resistance, suggesting a role for a soluble factor. Tinhofer et al. observed significant reduction in the pro-survival effect of CD4+ T cells in the presence of IL-4 blocking mAbs (30). Whether IL-4 plays a role in activated T cell induced drug resistance has to be determined. Blocking IL-2 or IL-21 did not have a significant effect on drug resistance nor on activation of CLL cells (data not shown).

Altogether, these data show that activation and drug resistance of CLL cells is induced in both in vitro systems, however the underlying mechanisms seem different. In the CD40L transfectant system the observed effects are obviously solely dependent on CD40L. In the activated T cell system the effects are only partly dependent on CD40L and other membrane-bound or soluble factors are likely to play a role. Reverse signaling via CD70 has been shown to support proliferation and cell cycle entry of B cells (31). CD70 expression on CLL cells is significantly upregulated on CLL after CD40 stimulation (12), suggesting a possible role for CD27-CD70 interaction in the CLL/activated T cell coculture. However, no effects were observed on activation or drug resistance of CLL cells when CD27-CD70 interaction was blocked (data not shown). Clearly, for both systems an upregulation of NF-κB induced anti-apoptotic genes was observed in the micro-array analysis, suggesting a common causal factor for drug resistance.

Several studies have shown that CLL is a dynamic disease with substantial proliferation rates (1, 2). However, in vitro CLL cells rapidly undergo apoptosis. Therefore, the in vitro system that closest resembles the in vivo situation must induce proliferation of CLL cells (next to activation and drug resistance). Our study shows for the first time that CLL cells cultured on CD40L transfectants and in the presence of IL-21 proliferate. Moreover, CLL cells cocultured with activated T cells proliferate in an IL-21- and CD40L- dependent manner. It has been shown that CLL cells upregulate IL-21R expression after CD40 stimulation (32). We confirmed this finding by FACS analysis (data not shown) and by micro-array analysis (Figure 5F). Interestingly, IL-21 has previously been shown to induce caspase-dependent apoptosis in CLL cells both in unstimulated (33) and CD40-stimulated CLL cells (32). Gowda also showed that IL-21 enhances apoptosis mediated by fludarabine and rituximab in unstimulated CLL (33). In contrast, our study shows proliferation of CLL cells in the presence of CD40L and IL-21. This difference can be explained by a different experimental setup; Totero induced apoptosis by sequential signalling of CD40L and IL-21 in contrast to simultaneous CD40L and IL-21 signaling resulting in proliferation of CLL cells in our study. In addition, differences in CLL cell density in the presence of IL-21 have an effect on viability (F. Pascutti, unpublished observation). Interestingly, both CD40L and IL-21 are required to induce proliferation suggesting the involvement
of complementary signalling pathways. Whether IL-21 also plays a role in inducing activation or drug resistance needs to be determined.

This study supports growing evidence for a key role of T cells in the pathogenesis of CLL in vivo. A significant increase in relative numbers of central and effector memory T cells was observed in the CD4+ T cell pool from CLL patients with unmutated IGHV genes as compared with patients with mutated IGHV genes and this was associated with high Rai stage, progressive disease and shorter treatment-free survival (30). Recently, Bagnara et al. elegantly showed in vivo proliferation of CLL cells under the influence of activated autologous CLL-derived T lymphocytes in a mouse model (34). There is also a clear role for regulatory T cells (Treg) in CLL. High Treg numbers are associated with unmutated IGHV genes, high Rai stage and poor clinical outcome (35) and we found evidence for CLL cell-induced Treg formation (36). In the present study CD4+ T cells, CD8+ T cells and possibly Tregs were present in the in vitro assays. The role of every individual subset in the induction of activation, drug resistance and proliferation of CLL cells in vitro has to be determined.

By inducing proliferation and drug resistance of CLL cells, activated autologous T cells might negatively affect the course of the disease (this study and (34)). On the other hand, increased T cell activation leads to an apparent graft-versus-tumor reaction in Bagnara’s mouse model(34). Also injection of autologous CLL cells transduced with CD40L leads to T cell activation and CLL cell elimination (37). Therefore, simple elimination of autologous activated T cells in the treatment of CLL will not result in a favourable outcome per se. Proliferation of CLL cells was dependent on simultaneous activation with CD40L and IL-21. Blocking CD40- and IL-21 signaling are likely to reduce proliferation of CLL cells in vivo, making these molecules interesting targets in the treatment of CLL.

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Reference List


