

FORMATION OF A TUMOR-SUPPORTIVE MICROENVIRONMENT IN CHRONIC LYMPHOCYTIC LEUKEMIA:

Addressing the reciprocal interactions in
the CLL—T cell—macrophage triad

M.H.A. VAN ATTEKUM

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About the cover: the cover depicts a sunny representation of a tumor microenvironment, where each umbrella represents a cell, colored by type. Communication between the umbrellas takes place via interactions between people (cytokines/receptors) that move from one umbrella to another over the sand (stroma). One umbrella habitat can furthermore support another by providing nutrients such as fresh lemonade to the other.

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INTRODUCTION

INTRODUCTION

The tumor microenvironment and chronic lymphocytic leukemia

In many types of cancers, the tumor microenvironment (TME) is increasingly recognized as a critical factor to drive tumor progression¹. Engagement with surrounding local cells can induce direct survival and therapy resistance of malignant cells, but can also aid indirectly in tumor support via mechanisms such as tissue remodeling, immunosuppression, angiogenesis and supporting tumor cell invasion^{1,2}.

Chronic lymphocytic leukemia (CLL) is considered a typical malignancy that depends on interactions with the lymph node (LN) microenvironment. CLL develops from a pre-malignant stage called monoclonal B cell lymphocytosis (MBL), which is characterized by low numbers of clonal B cells with a CLL-like phenotype³. MBL can be detected in approximately 3% of the general population over age 40 and progresses to CLL at a rate around 1-2% per year³. The pathogenesis of MBL and its progression towards CLL have not been fully elucidated, but a number of recurring genetic aberrations are considered to be drivers of the disease⁴. The affected genes play roles in several pathways, including inflammatory pathways (*MYD88*), B cell receptor signaling (*CARD11*), mRNA processing (*SF3B1*), and DNA damage control (*TP53* and *ATM*)⁴.

Although these findings strongly indicate that CLL pathogenesis is genetic, progression of the disease is thought to depend on microenvironmental factors. CLL cells are provided with essential survival and proliferative signals after interaction with bystander cells such as stromal cells, T cells and macrophages within the LN⁵. In addition, as malignant cells require changes in metabolism for proliferation and survival⁶, recent evidence suggests that TME signals could induce CLL cell metabolism⁷. The survival signals converge on B-cell lymphoma 2 (BCL-2) family members and LN-residing CLL cells have increased levels of these pro-survival proteins⁸. Next to these bystander cell-derived signals, B cell receptor activation in CLL cells provides survival and adhesion signals via downstream tyrosine kinases LYN, Spleen tyrosine kinase (SYK) and BTK⁹.

Mechanisms of CLL support by the TME

We have previously shown that T cell-mediated survival induction is largely governed via tumor necrosis factor (TNF) family member CD40L¹⁰ and that this effect depends on NF- κ B signaling⁸. Other survival-inducing factors secreted by T cells include interferon (IFN)- γ ¹¹ and interleukin (IL)-4¹². Next to their role in survival induction, T cells can induce antigen-independent CLL cell proliferation via CD40L in combination with IL-21¹⁰.

The contribution of monocyte-derived cells (MDCs) in CLL support is less well-known, but the level of circulating monocytes in CLL patients correlates with worse prognosis^{13,14}. The pivotal role of macrophages has recently been confirmed in a mouse study; the E μ -TCL1 mouse model for CLL, in which oncogene T-cell leukemia/lymphoma protein 1 (TCL1) is overexpressed under control of the B cell specific immunoglobulin heavy enhancer, develops clonal B lymphoproliferative disease at around 12 months of age¹⁵. Depletion of MDCs by treatment of these mice with clodronate containing liposomes led to a better overall survival¹⁶. Depending on the extracellular signals MDCs receive, they can differentiate to a number of phenotypes, including M1 (immunogenic) and M2 (tissue

repair) macrophages¹⁷. The supportive capacity of macrophages has been suggested to depend on this differentiation, as across different tumor types only an increased number of M2-skewed macrophages correlates with worse prognosis¹⁷. Several factors have been implicated in macrophage-mediated CLL cell survival. *In vitro*, CLL cell-differentiated monocytes (called Nurse-like cells, NLCs) can induce survival via cytokines such as CXC motif chemokine ligand (CXCL)12¹⁸, A Proliferation-Inducing Ligand (APRIL), and B-Cell-Activating Factor (BAFF)¹⁹. It has furthermore been shown that macrophages can induce CLL cell migration²⁰ and cause immune suppression^{16,21-24}. This latter effect is thought to occur via secretion of immunosuppressive cytokines such as IL-10²¹ but also via stimulation of immune checkpoint receptors such as Programmed cell death protein 1 (PD-1) on immune cells^{16,22-24}.

The CLL-T cell-macrophage triad

Next to the signals directed towards CLL cells, it has become evident that TME interactions are reciprocal in nature, and that signals derived from CLL cells could induce changes in bystander cells to drive tumor progression²⁵. Furthermore, CLL cells can coopt the physiological interactions taking place between immunological cells such as T cells and macrophages for their own benefit. The ensuing exchange of signals between CLL cells, T cells and macrophages, can thus lead to formation of a tumor-supportive triad between these cells in a mechanism called coevolution, which resembles the phenomenon occurring in nature²⁶.

Development of CLL therapies

Until recently, standard therapy for CLL was comprised of a combination of cytotoxic compounds fludarabine and cyclophosphamide with CD20 antibody rituximab (FCR)²⁷. Although FCR therapy leads to high response rates, it is not curative²⁷. Based on insights in the pathological mechanisms of CLL, several new compounds have been developed that are now being tested in clinical trials. First, as CLL cell survival induction is governed via upregulation of BCL-2 family members, compounds directed against these proteins have been developed, including venetoclax^{28,29}. Second, the recent insights into immune checkpoint activation via the PD-1/PD-L1 axis, has spurred the development of inhibitors directed against this axis, showing efficacy in the TCL1 mouse model³⁰. Third, as microenvironmental BCR signaling activates several survival pathways in CLL cells²⁵, targeted compounds against downstream kinases such as idelalisib³¹ and ibrutinib^{32,33} have shown promising results. It was recently found however, that the inhibition of BCR signaling via ibrutinib does not reduce cell survival, but rather reduces BCR-dependent cell adhesion resulting in CLL cell egress from the LN³⁴. These results verify the dependence of CLL cells on TME signals, and indicate that mechanistic knowledge of supportive signals could lead to the development of therapeutic compounds.

SCOPE

Despite recent advances in the understanding of the TME, the exact nature of TME interactions is at present however still not fully understood, particularly with respect to

MDCs. Moreover, most studies have addressed one-directional signals between bystander cells and CLL cells, but did not take into account the reciprocal signals between these cells. Lastly, the effects on CLL cell metabolism of these bystander cells have not been investigated.

In this thesis, we have therefore studied:

1. polarization of MDCs and recruitment towards the TME
2. the impact of MDCs on CLL cell survival and metabolism
3. whether T cells affect the interaction between MDCs and CLL cells
4. how reciprocal signals within this triad contribute to CLL support

In chapter 2, we investigate via which mechanisms monocytes are recruited towards the CLL TME. Using a novel overexpression coculture system, we then investigate in chapter 3 the contribution of APRIL in macrophage-mediated CLL cells survival. Our findings are then discussed in chapter 4 in light of the role of APRIL in normal B cell biology. In chapter 5, we compare the effects of macrophage and T cell signals on survival and the expression of BCL-2 family members in CLL cells. Next, we use gene expression profiling to mechanistically study these effects and explain the differences between macrophage and T cell effects. In chapter 6, the microarray analyses of the previous chapter are extended by studying whether macrophages influence metabolism in CLL. Lenalidomide is a clinically effective drug with several immunomodulatory effects, including effects on macrophages. Although it has no cytotoxic effects on CLL cells, recent findings indicate that its efficacy could also result from other effects on CLL cells, which we investigate in chapter 7. Lastly, we review our own findings in context of current literature in chapter 8 to explore how interactions between different bystander cells contribute in the formation of a supportive microenvironment, focusing on the role of the CLL cell.

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CD40 SIGNALING INSTRUCTS CHRONIC LYMPHOCYTIC LEUKEMIA CELLS TO ATTRACT MONOCYTES VIA THE CCR2 AXIS

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ABSTRACT

Chronic lymphocytic leukemia (CLL) cells are provided with essential survival and proliferative signals in the lymph node (LN) microenvironment. Here, CLL cells engage in various interactions with bystander cells such as T cells and macrophages. Phenotypically distinct types of tumor infiltrating macrophages can either be tumor supportive (M2) or play a role in tumor immune surveillance (M1). Although recent *in vitro* findings suggest a protective role for macrophages in CLL, the actual balance between these two types of macrophage subsets in CLL lymphoid tissue is still unclear. We here address that question and also the mechanism of recruitment of monocytes towards the CLL LN.

Immunofluorescence staining of LN samples showed skewing towards an M2 tumor-promoting phenotype. This polarization likely results from CLL-secreted soluble factors, as both patient serum and CLL-conditioned medium recapitulated the skewing effect.

Considering that LN-adjacent T cells affect CLL cell cytokine expression and secretion, we next studied CLL-mediated monocyte recruitment in the presence or absence of T cell signals. While unstimulated CLL cells were inactive, T cell-stimulated CLL cells actively recruited monocytes. This correlated with secretion of various chemokines such as CCL2,3,4,5,7,24, CXCL5,10, and IL-10. We furthermore identified CD40L as the responsible T cell factor that mediated recruitment and showed that recruitment critically depended on the CCR2 axis.

These studies show that a triad of interactions between CLL cells, T cells and monocytes can shape a beneficial tumor microenvironment and that this is CCR2 dependent. Therefore, targeted inhibition of CD40L or CCR2 may be relevant therapeutic options.

INTRODUCTION

Chronic lymphocytic leukemia CLL cells strongly depend on interactions with bystander T cells and monocyte derived cells (MDCs) within the lymph node (LN) microenvironment for their survival and resistance to therapy¹. The role of LN-residing T cells in the pathogenesis of CLL has gained much attention. It is suggested that interaction between neoplastic B cells with T cells results in skewing of the T cell compartment towards CD40L-expressing CD4⁺ T cells². These T cells in turn induce both CLL cell survival and proliferation via upregulation of several pro-survival molecules as well as increased secretion of cytokines^{3,4}. The interaction between MDC's and CLL is less understood, although *in vitro* experiments show that MDCs -in the form of Nurse like cells- can induce CLL cell survival⁵ through C-X-C motif chemokine 12, B-cell activating factor and A proliferation-inducing ligand signaling^{5,6}.

Based on data from different malignancies, two subgroups of tumor-associated macrophages (TAMs) exist: M2-like CD68⁺CD163⁺/CD206⁺ macrophages are characterized by an immunosuppressive phenotype, whereas M1-like CD68⁺CD80⁺ macrophages display an immune-surveilling phenotype⁷. Although large intratumoral and intertumoral heterogeneity exists, it has been suggested that M1 TAMs lead to a better and M2 TAMs lead to a worse prognosis across different tumor types⁸. Tumors that are associated with M2 TAMs include breast⁹, ovarian⁷, prostate¹⁰ cancer, whereas colon carcinoma TAMs are of M1 phenotype¹¹.

With respect to CLL, *ex vivo* evidence shows that MDCs are present in the LN¹², and it was recently shown that MDCs contribute to CLL progression as MDC depletion by clodronate treatment in the TCL1 CLL mouse model leads to slower CLL progression^{13,14}. Whether LN-residing macrophages in human CLL are indeed of a protective M2 phenotype has however not been directly studied. It is furthermore unknown whether circulating monocytes can actively be recruited towards the tumor-infiltrated LN.

Migration of CLL cells to the LN microenvironment depends on chemotactic gradients through the CXCL12/CXCR4¹⁵, CXCL13/CXCR5¹⁶ and CCL19,21/CCR7¹⁷ axes. Upon interaction with LN-residing cells such as T cells, CLL cells can alter their secretome^{4,18,19}, which in turn could potentially impact both skewing and migration of other cells like MDC's. Co-operative or reciprocal signals between the triad formed by CLL cells, T cells, and macrophages could therefore critically contribute to the supportive microenvironment for CLL cells.

Here, we investigated both the possibly supportive differentiation of MDCs and their recruitment as a result of CLL-secreted cytokines in the context of T cell signals. We found that CLL-secreted factors were able to differentiate macrophages towards a supporting M2 phenotype. Secondly, T cell/CD40 stimulation of CLL cells induced CLL cells to recruit monocytes which critically depends on CCR2 signaling.

RESULTS

2

LN-residing macrophages are of M2 phenotype, and CLL cells and serum induce M2 skewing

To study the phenotype of macrophages in the CLL LN, paraffin-embedded LN sections from CLL patients were stained for the pan-macrophage marker CD68 in combination with either the M1 marker CD80 or the M2 marker CD206 using immunofluorescence. The CD80/CD206 fluorescence signal per macrophage (CD68⁺) was then quantified using an automated cell identification pipeline in CellProfiler. CD68 positive cells were present in all samples tested and were dispersed throughout the CLL-infiltrated LNs (Figure 1A-B). In these CD68⁺ cells a higher CD206 intensity was observed as compared to CD80 (0.87 versus 0.45 arbitrary units; Figure 1A-C and S1).

In order to study whether the leukemic cells could account for the observed M2 polarization, we first tested whether soluble factors present in CLL serum differentiated monocytes towards an M2 phenotype. Freshly isolated monocytes from HDs were incubated with either sera from 25 different CLL patients or pooled serum from HDs and differentiation status was measured using flow cytometry. IFN- γ (M1) and IL-4 (M2) differentiated monocytes were included for comparison. Both M2 markers CD163 and CD206, but not M1 marker CD80, were increased in CLL serum-differentiated monocytes compared to HD serum-differentiated monocytes (Figure 1D).

As CLL-serum components resulted in M2 differentiation, we next investigated whether the observed M2 differentiation in the LN was actuated by CLL cells. To this end, healthy donor (HD)-isolated monocytes were differentiated for 72h using CLL cells or positive control NAMPT²⁰. IFN- γ (M1) and IL-4 (M2) differentiated monocytes were again included as control. We found an upregulation of M2 markers after IL-4 stimulation. In line with the differentiation effect of CLL serum, both CLL cells and NAMPT induced an upregulation of the M2 markers, but not of the M1 marker (Figure 1E). Furthermore, the M2 differentiation depended on soluble factors, as conditioned medium from CLL cells likewise induced M2 differentiation (data not shown).

These data together indicate that CLL-secreted factors are able to differentiate macrophages towards an M2 phenotype.

T cell-stimulated CLL cells secrete monocyte-attracting chemokines

Next, we investigated whether CLL cells could direct monocyte migration. Using trans-well migration assays, we found no migration of HD monocytes towards supernatants of unstimulated CLL cells. CLL cells interact with residential T cells, such as follicular helper T cells, within the LN²¹ (Figure 1A) which might affect CLL cytokine secretion. Therefore, supernatants of CLL cells cultured with unstimulated or α CD3/ α CD28 activated autologous T cells (T_{act}) were tested for induction of monocyte recruitment. Only medium from CLL cells co-cultured with activated T cells induced migration of monocytes (Figure 2A).

To determine the candidate chemokines expressed by stimulated CLL cells that could underlie the recruitment of monocytes, we analyzed our previously generated microarray dataset (GSE50572) of purified CLL cells that were stimulated with T_{act}⁴. Expression of several monocyte-attracting chemokines such as CCL2, 3, 4, 5, 7, CXCL1, 5, 10 and

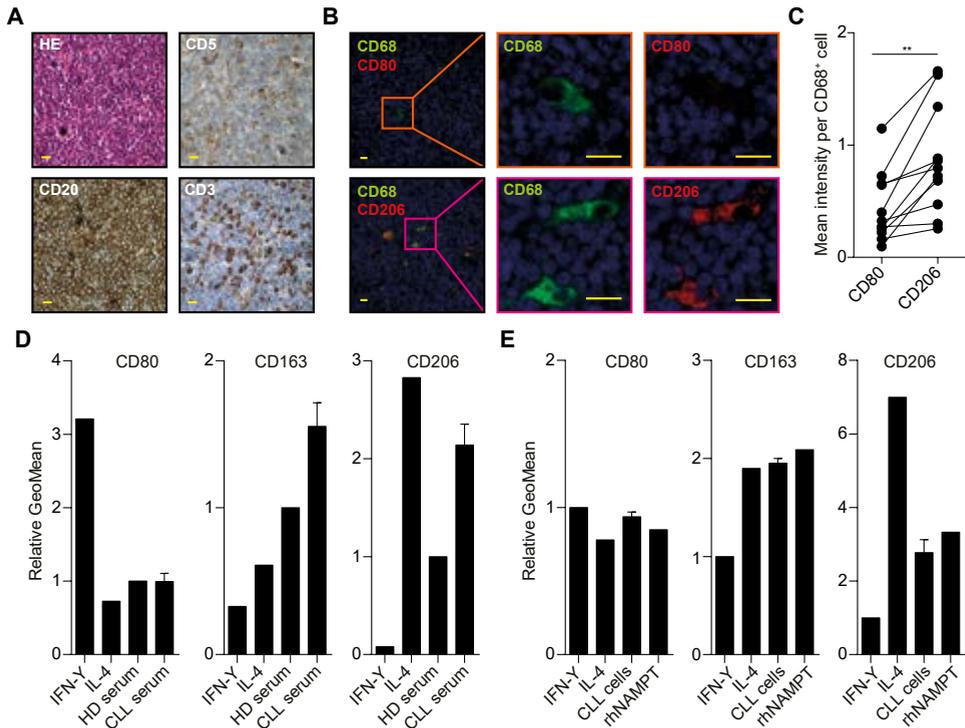


Figure 1: CLL cells differentiate monocytes towards an M2 phenotype. **A.** Paraffin-embedded LN material from CLL patients (N=11) was stained by immunohistochemistry for CLL markers CD5 and CD20 and T cell marker CD3. Shown is one representative slide. Yellow scale bars correspond to 20 μ m. More information on image acquisition can be found in the methods section. **B.** The CLL slide shown in Figure 1A was stained by immunofluorescence for pan-macrophage marker CD68 in combination with either M1 marker CD80 or M2 marker CD206. Yellow scale bars correspond to 20 μ m. **C.** Each CLL slide was stained as in Figure 1B and subjected to automated image analysis (see methods) measuring the intensity of the M1/M2 marker (CD80/CD206, both red) signal per macrophage (CD68, green). Per-patient (each line) average macrophage intensity of both CD80 and CD206 are indicated (each dot). **, $P < .01$ in a paired t-test. **D.** HD monocytes were differentiated for 72h with IMDM containing 25% CLL serum or 25% pooled HD serum, or with complete medium containing IFN- γ (M1) or IL-4 (M2) as controls. Their differentiation was then tested as in Figure 1D. Each bar represents the relative geometrical mean (GeoMean) of the fluorescence signal compared to the HD serum condition and error bars indicate SEM of N=25 CLL serum samples. **E.** HD monocytes were differentiated with CLL cells in complete medium, or with complete medium containing IFN- γ (M1), IL-4 (M2) or recombinant human (rh)NAMPT as controls. Their differentiation was then tested after 72h by staining for M1 marker CD80 and M2 markers CD163 and CD206 using flow cytometry. Each bar represents the relative geometrical mean (GeoMean) of the fluorescence signal compared to the M1 condition and error bars indicate SEM of N=3 CLL samples.

IL-10²²⁻²⁵ was upregulated in CLL cells after contact with T_{act} (Figure 2B). To measure chemokine secretion by CLL cells, a Luminex assay was performed on the conditioned media used in Figure 2A. All chemokines that were upregulated on mRNA level, were also significantly upregulated on protein level (Figure 2C).

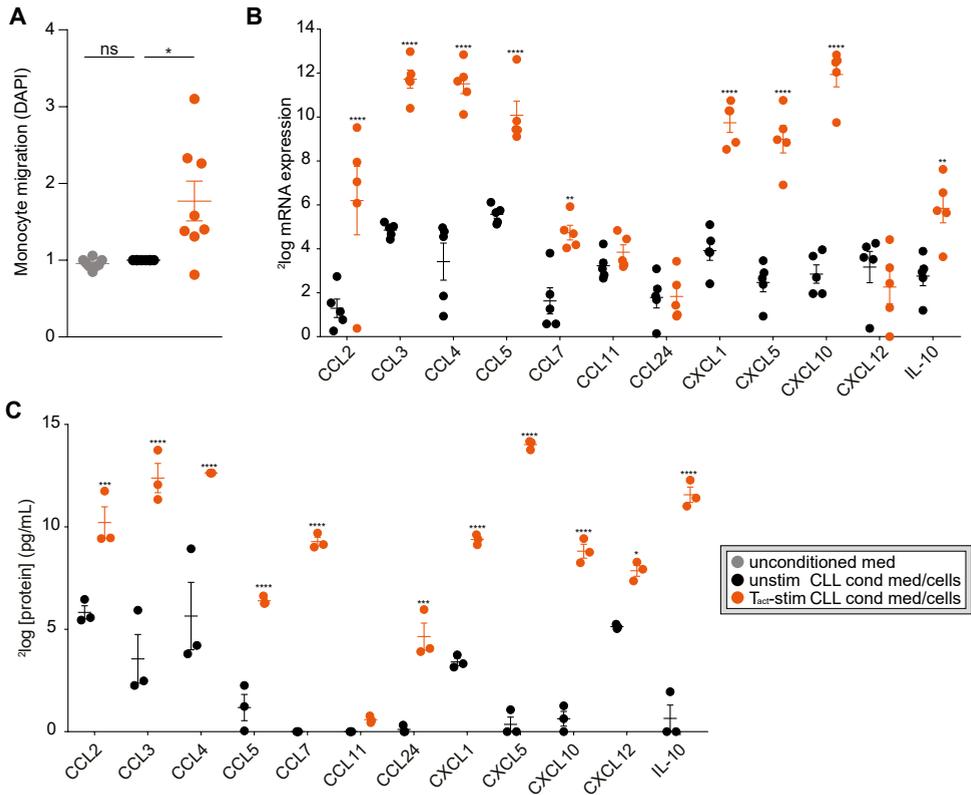


Figure 2: T cell-stimulated CLL cells secrete monocyte-attracting chemokines. **A.** Freshly isolated HD monocytes were seeded in the upper chambers of a trans-well migration plate to migrate towards conditioned media obtained from PBMC samples from CLL patients (for characteristics see table S1) that were unstimulated or stimulated for 72h with HD PBMC T cells that were activated using α -CD3/ α -CD28 antibodies. Next, the amount of migrated monocytes was quantified using DAPI staining. Each dot represents the relative (compared to the unstimulated CLL condition) DAPI signals of 8 different CLL conditioned media or 3 control media in 3 independent experiments using monocytes from 3 different donors and mean \pm SEM are shown. All measurements were performed in triplicate. *, $P < .05$ in t-tests. **B.** CLL cells were stimulated with α -CD3/ α CD28 activated T cells or not stimulated for 16h. RNA from CD5/CD19 FACS sorted CLL cells (>99% purity) was subjected to microarray analysis and tested for differential expression of chemokines involved in monocyte migration²²⁻²⁵. Dots represent expression levels and mean \pm SEM are shown for 5 paired CLL samples. **, $P < .01$; ****, $P < .0001$ in a two-way ANOVA test with Bonferroni post-hoc analysis. **C.** Protein levels of chemokines involved in monocyte migration²²⁻²⁵ were determined in the conditioned media that were used to perform the migration assays in Figure 2A by using Luminex. Dots represent protein levels and mean \pm SEM are shown for 3 CLL conditioned media; *, $P < .05$; ***, $P < .001$, ****, $P < .0001$ in a two-way ANOVA test with Bonferroni post-hoc analysis.

CD40L-stimulated CLL cells attract monocytes as a result of CCR2 axis signaling

As T_{act}-stimulated CLL cells have highly similar gene expression profiles compared to CD40L-stimulated CLL cells⁴, we investigated if CD40L stimulation similarly endows CLL

cells with monocyte recruiting capacity. Comparable to the T_{act} results, supernatants from CD40L-stimulated CLL cells induced migration of monocytes (Figure 3A). These data indicate that a co-operative signal from T_{act} cells is needed for CLL cells to induce monocyte migration. Furthermore, CD40L appears to be responsible for the T_{act} -mediated monocyte migration. Of note, by using this T cell free CD40L system, these data indicate that CLL- (rather than T_{act} -) derived chemokines induce recruitment of monocytes. Secreted proteins in the conditioned media from CD40L-stimulated CLL cells were measured. In line with the T_{act} data, several monocyte-attracting chemokines such as CCL2, 3, 4, 5, 7, 24, CXCL5, 10 and IL-10 were secreted by the CLL cells after CD40L stimulation (Figure 3B). None of the chemokines tested was detectable in supernatant from CD40L overexpressing NIH-3T3 cells alone (data not shown).

To pinpoint which of the upregulated candidate chemokines was responsible for the migration of monocytes, we applied selective small molecule inhibitors for the relevant chemokine receptors²²⁻²⁵. Inhibition of CCR2 was sufficient to reduce migration to background level. There was no additive effect of inhibition of other chemokine receptors, as a combination of the different receptor inhibitors yielded similar inhibition as CCR2 inhibition alone (Figure 3C). In a control experiment no direct cytotoxic effect of the CCR2 inhibitor was detected after 72h stimulation of CLL cells (Figure S2). Furthermore, supernatants from unstimulated CLL cells in combination with the different chemokine receptor inhibitors showed migration comparable to background (data not shown).

As potent CCR2 ligand, we next applied recombinant CCL2²⁶, which resulted in monocytes migration (Figure 3D). Combined, these data suggest that CD40 signaling is responsible for T cell-mediated monocyte migration by CLL cells and that this migration depends on the CCL2-CCR2 axis.

DISCUSSION

It is widely accepted that interactions with local bystander cells in the LN are critical for CLL maintenance¹. Various reports have mechanistically elucidated how bystander cells can support CLL cells, but the active role of CLL cells in shaping this supportive microenvironment is still largely unclear. In this complex interplay between the leukemic and various types of surrounding cells, we functionally addressed two key aspects: the chemo-attraction of monocytes, and the cross-talk between CLL cells and activated T cells herein. Our findings are compatible with a model (Figure 4) in which stimulation by CD40L on T cells in the LN induces CLL cells to secrete several monocyte-attracting chemokines. Of these, CCL2 can subsequently signal via CCR2 on monocytes to recruit them towards the malignant cells in the LN. The immuno-fluorescence data suggest that following engagement with CLL cells in the LN, monocytes undergo skewing towards a tumor-supportive M2 phenotype (see also below).

Based on several reports that studied migration of monocytes in the context of inflammation, chemo-attraction can occur via activation of several different chemokine receptor signaling pathways²²⁻²⁵. We here identified CCR2 as the most likely responsible receptor in the context of monocyte recruitment towards the CLL LN. The most potent chemokine that recruits monocytes via CCR2 receptor is CCL2²⁶, which indeed recruited monocytes in our experiments (Figure 3D). These data are in line with the recent observation

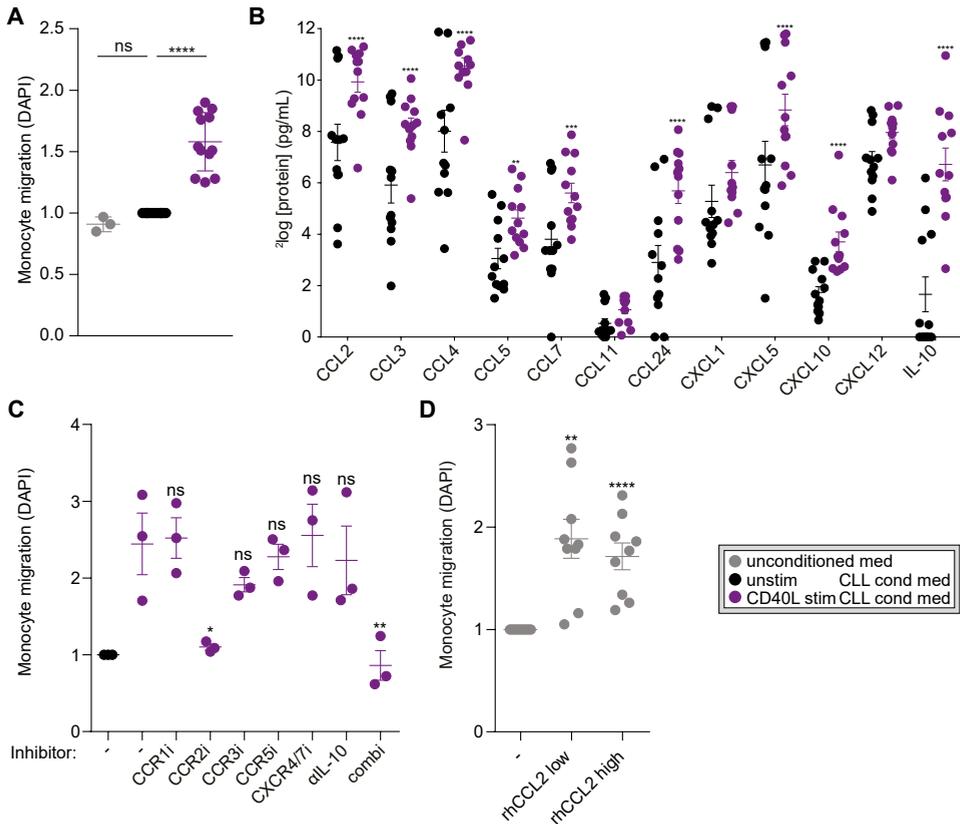


Figure 3: CD40L-stimulated CLL cells attract monocytes as a result of CCR2 axis signaling. **A.** Freshly isolated HD monocytes were seeded in the upper chambers of a trans-well migration plate to migrate towards conditioned media obtained from PBMC samples from CLL patients (for characteristics see table S1) that were cultured for 16h on CD40L-overexpressing (CD40L-stim) or parental NIH-3T3 cells (unstim). Next, the amount of migrated monocytes was quantified using DAPI staining. Each dot represents the relative (compared to the unstimulated CLL condition) DAPI signals of 12 different CLL conditioned media or 3 control media in 3 independent experiments using monocytes from 3 different donors and mean \pm SEM are shown. All measurements were performed in triplicate. ****, $P < .0001$ in t-tests. **B.** Protein levels of chemokines involved in monocyte migration²²⁻²⁵ were determined in the conditioned media that of Figure 3A by Luminex. Dots represent protein levels and mean \pm SEM are shown for 12 CLL conditioned media; **, $P < .01$; ***, $P < .001$ in a two-way ANOVA test with Bonferroni post-hoc analysis. **C.** Freshly isolated monocytes and pooled conditioned media collected for Figure 3A were pre-incubated for 30 min with individual small-molecule inhibitors directed against indicated chemokine receptors, with an IL-10 neutralizing antibody, or a combination of all inhibitors (combi), before performing migration assays as in Figure 3A. Each dot represents the relative (compared to the unstimulated CLL condition) DAPI signals of 3 independent experiments using monocytes from 3 different donors and mean \pm SEM are shown. All measurements were performed in triplicate. *, $P < .05$; **, $P < .01$; in a one-way ANOVA test with Bonferroni post-hoc analysis. **D.** Monocytes were seeded in the upper chambers of a trans-well migration plate to migrate towards migration medium without or with 10 ng/mL recombinant human CCL2 (rhCCL2 low) or 100 ng/mL rhCCL2 (rhCCL2 high). Next, the amount of migrated monocytes was quantified using DAPI staining. Each dot represents the relative (compared to the unstimulated CLL condition) DAPI signals of 9 separate read-outs in 3 independent experiments using monocytes from 3 different donors and mean \pm SEM are shown. **, $P < .01$; ****, $P < .0001$ in t-tests.

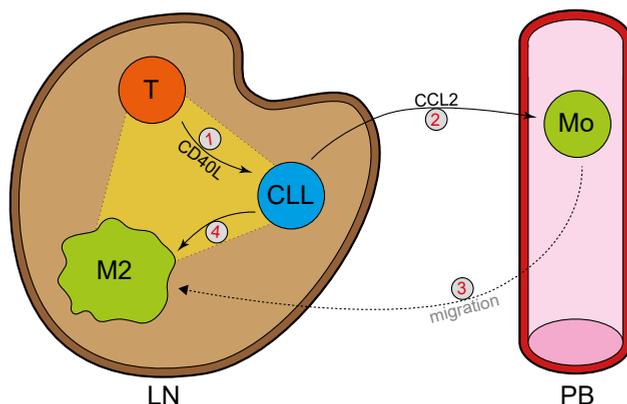


Figure 4: Model of CLL - T cell - macrophage triad in the formation of a supportive tumor microenvironment. Stimulation of CLL cells by activated T cell-produced CD40L (1) induces them to secrete CCL2 (2), which in turn recruits monocytes towards the LN (3). As a result of CLL-secreted factors, monocytes differentiate towards a tumor supporting M2 phenotype (4).

that adoptive transfer of leukemic TCL1-derived splenocytes into recipient mice that are deficient for the CCL2 receptor CCR2 resulted in significantly lower percentages and numbers of monocytes in the spleen¹³.

Besides its importance in CLL, CCL2 has been shown to recruit monocytes towards primary tumors in prostate cancer. This recruitment furthermore resulted in enhanced tumor growth²⁷. CCR2 antagonist PF-04136309 reduced the number of monocytes and restored chemo-sensitivity in a pancreas tumor mouse model, indicating the therapeutic potential of CCL2/CCR2 inhibition²⁸. Our studies suggest that also in CLL these inhibitors can be a relevant therapeutic option.

In light of the large number of potential interactions in the CLL LN, it is worth noting that specifically the T cell co-stimulatory signal CD40L leads to induction of monocyte trafficking. The levels of chemokines secreted by unstimulated CLL cells are insufficient to induce migration above background (Figure 2A and 3A). Although CLL cells stimulated by monocyte-derived Nurse-like cells show increased production of CCL3 and CCL4¹⁹, these cytokines apparently play a subordinate role in monocyte recruitment: despite their presence in the conditioned media (Figure 3B), monocyte migration is not prevented by blocking their cognate receptors CCR1 or -5 (Figure 3C). In contrast to the monocyte-attracting effect by CLL cells, it has been shown that bystander cells such as CD3⁺ or CD68⁺ cells are unable to produce CCL2 themselves²⁹. We have previously shown that CD40L accounts for most of the transcriptional effects of T cells on CLL cells⁴ and based on our data, CD40L is sufficient to induce CCL2 production and monocyte recruitment. In this context, others have shown that another key T cell cytokine –IL21– is dispensable for CCL2 induction³⁰.

Our observation that the large majority of macrophages in the CLL LN are of an M2 phenotype (Figure 1B,C) strongly suggest initiation of M2 differentiating signaling events once monocytes enter the CLL lymph node environment. Factors that can account for this differentiation include NAMPT²⁰ or High mobility group box 1 (HMGB-1)¹² secreted by

LN-residing CLL cells. We could confirm that addition of NAMPT indeed skews monocytes towards an M2 type (Figure 1D). In addition, T helper-2 cells that also reside in the LN²¹ secrete various cytokines that induce M2 differentiation, including IL-4, IL-10, and IL-13. Of note, the production of IL10 could be complemented by CLL cells that are stimulated by T cells (Figure 2C, 3B). This indicates that the LN provides an M2-inducing milieu, which likely results in a supportive macrophage phenotype that can induce CLL cell survival and immune-suppression.

Indeed, the association of M2 differentiation and tumor support has been pointed out in several other tumor types⁸⁻¹¹. Functionally, the tumor-promoting effects of M2 macrophages have been attributed to an increased production of direct tumor-promoting cytokines³¹ and a suppression of the immune response²⁰. The factor responsible for macrophage skewing differs between tumor types, although lactic acid -that is produced as a result of increased glycolysis in several different tumors- could induce M2 differentiation in multiple tumor types³².

Besides their direct tumor promoting effects, M2 macrophages can furthermore induce a suppression of cytotoxic T cells, as they can induce expression of PD-1 on T cells²⁰. In addition, they inhibit T cell proliferation²⁰. Lastly, M2 macrophages suppress T cell activation and promote the differentiation towards T_{reg} cells³³. In light of the recent development of T cell therapy against CLL neoantigens³⁴, the subversion of T cells by macrophages is an important point to address.

In conclusion, our studies provide insight in several aspects of the complex interactions that take place in the CLL LN and indicate how the triad of CLL cell, T cell, and macrophage contributes to the shaping of the tumor-microenvironment in CLL. Finally, we identified CCR2 as a potential therapeutic target to interrupt the intercellular interplay.

METHODS

Patient samples, stimulation and conditioned medium collection

Patient material was obtained from CLL patients, after written informed consent according to the guidelines of the Medical Ethical Committee of the Academic Medical Center, Amsterdam, following the Declaration of Helsinki protocols. For T cell stimulation, peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors (HDs) using ficoll gradient purification according to manufacturer's instructions (Lucron, Dieren, The Netherlands). When cultured without T cells, CLL cells were used at a concentration of $1,5 \times 10^6$ cells/ml. When cultured with T cells, PBMCs were added to CLL cells in a 1:1 ratio at a concentration of 1×10^6 cells and stimulating antibodies directed against CD3 (1 μ g/mL, clone 1XE, Sanquin, Amsterdam, The Netherlands) and CD28 (3 μ g/mL, clone 15E8, Sanquin) were added to activate the T cells. After 72h, conditioned medium was collected. For stimulation with CD40L, CLL cells were cultured on CD40L transfected NIH-3T3 cells generated as described before³, or on control 3T3 cells, all in IMDM supplemented with 10% Fetal Bovine Serum (Invitrogen, Carlsbad, CA, USA), 100U/mL Penicillin-100 μ g/mL Streptomycin (Life Technologies, Austin, TX, USA), 2 mM L-glutamine (Life Technologies) and 0.00036% β -mercaptoethanol (Sigma, St. Louis, MO, USA) (IMDM^{+/+}) for 16h after which conditioned medium was collected. Cell-free conditioned medium was kept at -80°C until use.

Migration assays

Conditioned or control media were diluted 1:2 in chemotaxis medium (PBS with 1% albumin, low endotoxin, Sigma). Monocytes were freshly isolated from HDs after obtaining written informed consent using negative MACS depletion as described previously³⁵ and resuspended in chemotaxis medium. When applicable, both media and monocytes were incubated for 30 min on ice with the indicated inhibitors before the diluted conditioned media was added in the lower chambers of a 5 μ m chemotaxis assay plate (96 well ChemoTX®, NeuroProbe, Gaithersburg, MA) and 100,000 monocytes were transferred to the upper chamber. After 2h, chemotaxis was quantified by measuring the DAPI (4,6 diamidino-2-phenylindole) signal of migrated monocytes as described before³⁵.

Chemokine production and inhibitor experiments

Previously generated microarray profiles⁴ of purified (>99%) CLL cells stimulated for 16h with activated T cells (deposited under accession number GSE50572) were normalized and analyzed using the R2 platform (<http://r2.amc.nl>) and extracted using its DataGrabber feature. When testing protein secretion, conditioned media were analyzed for the indicated chemokines by Luminex using the ProcartaPlex 9-plex chemokine immunoassay kit extended with CCL7, CCL24, CXCL5, and IL-10 (eBioscience, San Diego, CA, USA) according to manufacturer's instructions. In chemotaxis assays, the following chemokine receptor inhibitors were used: 1 μ g/mL CCL1 inhibitor BX471 (Sigma), 1 μ g/mL CCR2 inhibitor INCB3284 (Tocris Bioscience, Bristol, UK), 1 μ M CCR3 inhibitor SB328437 (Tocris), 1 μ M CCR5 inhibitor Maraviroc (Apexbio, Houston, TX, USA), 1 μ M CXCR4/7 inhibitor Plerixafor (Apexbio), and 0.1 μ g/mL IL-10 neutralizing antibody (R&D systems, Minneapolis, MN, USA).

Monocyte isolation and *in vitro* differentiation

Monocytes were obtained by isolation from HDs after obtaining written informed consent. To this end, PBMCs were isolated using ficoll gradient purification according to manufacturer's instructions, after which monocytes were separated from peripheral blood lymphocytes using percoll gradient purification (GE healthcare, Milwaukee, USA). Next, monocytes were incubated to adhere at 37°C in 5% CO₂ for 40 min at a concentration of 0.75*10⁶ cells/mL in 6-well plates (3 mL) in IMDM supplemented with 1% Fetal Bovine Serum and washed to remove non-adherent cells. The monocytes were then differentiated using CLL cells in IMDM^{+/+} or 200 ng/mL NAMPT in IMDM^{+/+} for 72h. Alternatively, monocytes were differentiated using IMDM^{-/-} supplemented with 25% serum from different CLL patients or 25% pooled serum from HDs (Human Serum Type AB, Sigma) for 72h. Control monocytes were differentiated to either M1 using 10 ng/mL IFN- γ or M2 using 10 ng/mL IL-4 (both R&D systems, Minneapolis, MN, USA) in IMDM^{+/+}. Next, the differentiated macrophages were removed from the plates using 80 mM Lidocaine (Sigma) in PBS/10 mM EDTA (Merck, Darmstadt, Germany). They were then stained for the indicated markers using CD80-FITC (eBioscience), CD163-PE (Beckton Dickinson Biosciences [BD], San Jose, CA), CD206-APC (BD), or relevant isotype controls, after which fluorescence signals were measured on a FACS Canto II (BD). Analysis was then performed using FlowJo software

(TreeStar, San Carlos, CA, USA). For cell viability, cells were analyzed by flow cytometry using a Dioc6-PI staining as described before³⁶.

LN material and Immunofluorescence

Four-micron sections from paraffin-embedded whole LN extirpations (n=11) were obtained from the AMC Pathology department. All material was derived from either untreated patients or patients at least 3 months after chemotherapy. No patients received kinase inhibitor therapy. Localization of CLL was proven by standard CD5/CD19/CD20/CD3 immunohistochemistry as performed by standard diagnostic pathology work-up. To test macrophage differentiation, sections were de-waxed by immersion in xylene and hydrated by serial immersion in ethanol and PBS. Antigen retrieval was performed by heating sections for 20 min in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween20, pH 6.0). Sections were washed with PBS (2 x 10 min) and blocking buffer (TBS containing 10% BSA and 0.3% Triton X-100) was added for 1h. Sections were incubated with primary antibody, pan-macrophage marker CD68 (clone PG-M1, DAKO, Carpinteria, CA, USA) in combination with either M1 marker CD80 (ab134120, Abcam, Cambridge, MA, USA) or M2 marker CD206 (ab64693, Abcam) in SignalStain (Cell Signaling) or blocking buffer, respectively, overnight at 4°C. Subsequently, the slides were washed with PBS (2 x 10 min) and incubated with goat anti-rabbit Alexa594 or goat anti-mouse Alexa488 (both Life Technologies, Carlsbad, CA, USA) for 1h, after which the slides were stained for 10 minutes with DAPI (0.1 µg/ml in PBS). Sections were mounted with Fluoromount-G (eBioscience) and immunofluorescent imaging was performed using a Leica DMRA fluorescence microscope equipped with a cooled camera. Images were acquired using Image Pro Plus and composed in Adobe Photoshop CS3. For signal quantification, a Cell Profiler (<http://cellprofiler.org>) pipeline was created to measure the red intensity (CD80/CD206) of green cell objects (CD68), using "IdentifyPrimaryObjects" for identification with automatic thresholding and "shape" as distinction method. Averages of these per-cell intensities were subsequently calculated in R (<https://www.r-project.org/>).

Statistical analysis

Two-way analysis of variance (ANOVA) with Bonferroni post-hoc tests were performed to test for significant differences between multiple groups using Graphpad Prism software (Graphpad, La Jolla, CA, USA). A (paired) t-test was used to test for significant differences between two groups. *P* values <.05 (*), <.01 (**), <.001 (***) and <.0001 (****) were considered statistically significant. Error bars represent standard error of the mean (SEM).

AUTHOR CONTRIBUTIONS

MHAvA designed and performed experiments and wrote the manuscript. MCL aided with and designed migration experiments, ES designed and performed immunofluorescence experiments, ER performed differentiation experiments, SK provided patient samples and scientific input, EE and APK supervised the study and wrote the manuscript.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIALS

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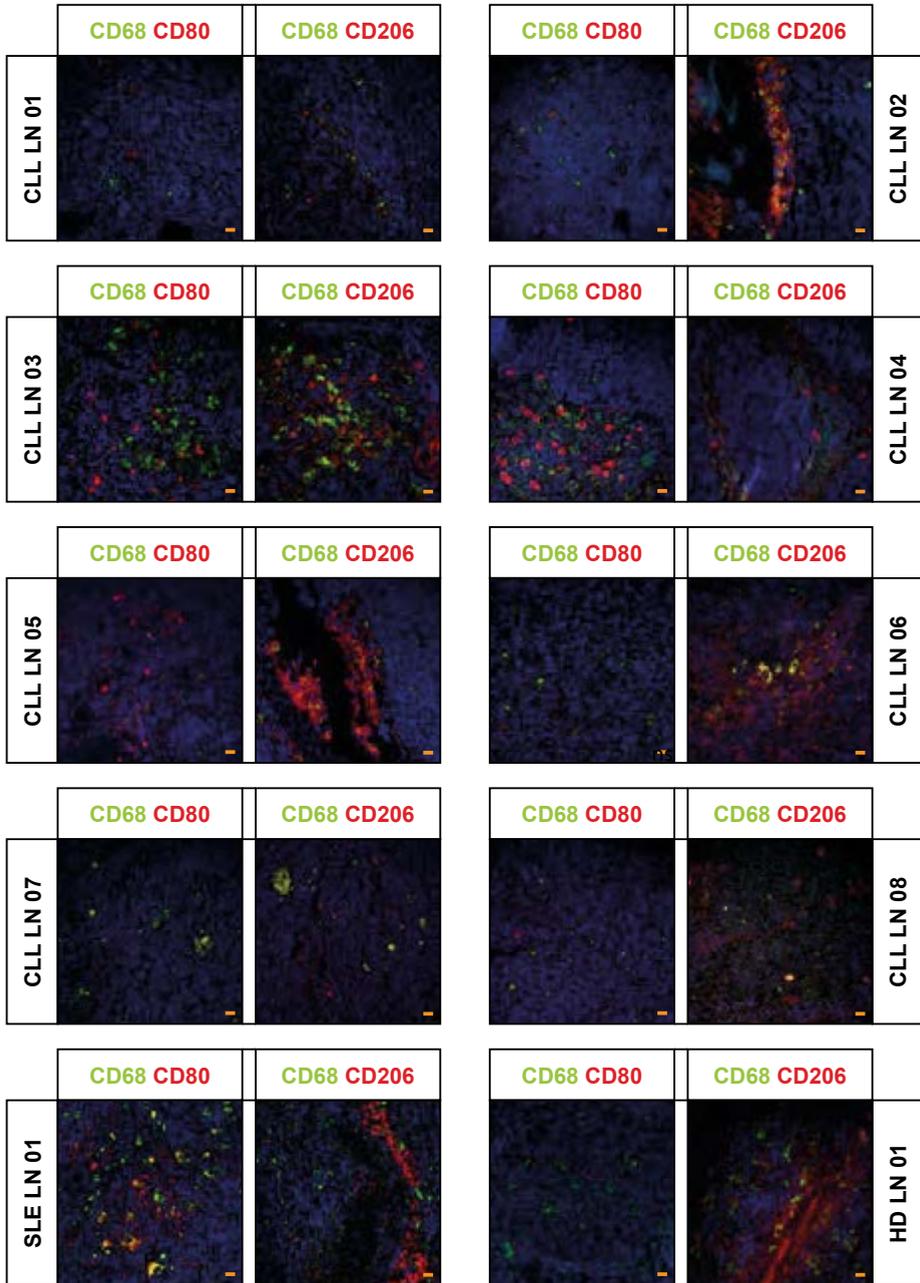


Figure S1: Additional Immunofluorescence stainings for macrophage differentiation markers. 8 additional CLL LN, 1 control Systemic lupus erythematosus (SLE), and 1 HD reactive LN slides were stained as in Figure 1B. Yellow scale bars correspond to 20μm. Note the CD206⁺ high endothelial venule structures in a few sections.

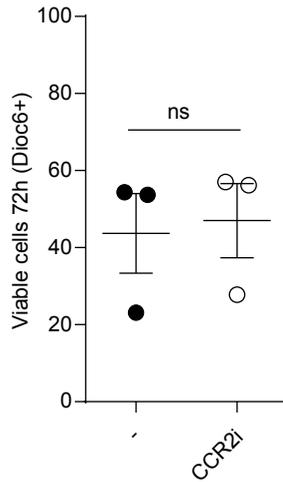


Figure S2: The CCR2 inhibitor INCB3284 has no cytotoxic effect on CLL cells after extended culture. CLL cells were cultured in the presence or absence of 1 μ g/mL CCR2 inhibitor INCB3284 and viability was measured after 72h using a Dioc6-PI staining. ns, not significant using a paired t-test.

Table S1: Characteristics of CLL patients that provided samples for this study

ID#	Sample#	Age (years)	WBC count $\times 10^9/L$	RAI CD3 (%)	RAI stage	IgVH status	Chromosomal aberrations	Last therapy
1	1407	78	216	1,4	n.d.	n.d.	n.d.	none
2	1408	63	161	3,3	0	n.d.	n.d.	none
3	1465	65	124	2,6	0	M	13q-	none
4	1177	75	29	5,7	0	M	none	none
5	1200	77	28	5,0	0	n.d.	n.d.	none
6	1266	74	17	6,6	0	n.d.	n.d.	none
7	1314	77	28	4,9	0	n.d.	n.d.	none
8	1322	87	39	7,9	0	n.d.	11q-	none
9	1353	86	28	5,3	n.d.	M	none	Chlorambucil
10	1357	76	38	7,9	0	M	n.d.	none
11	1365	73	34	10,9	1	n.d.	n.d.	Chlorambucil
12	1605	68	21	4,9	0	n.d.	n.d.	unknown

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MACROPHAGE-MEDIATED CHRONIC LYMPHOCYTIC LEUKEMIA CELL SURVIVAL IS INDEPENDENT OF A PROLIFERATION INDUCING LIGAND (APRIL) SIGNALING

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ABSTRACT

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Survival of chronic lymphocytic leukemia (CLL) cells is mainly driven by interactions within the lymph node (LN) microenvironment with bystander cells such as T cells or cells from the monocytic lineage. While the survival effect by T cells is largely governed by the TNFR ligand family member CD40L, the exact mechanism of monocyte-derived cell-induced survival is not known. An important role has been attributed to the TNFR ligand A Proliferation Inducing Ligand (APRIL), although the exact mechanism remained unclear. Since we detected that APRIL was expressed by CD68⁺ cells in CLL LN, we addressed its relevance in various aspects of CLL biology, using a novel APRIL overexpressing co-culture system, recombinant APRIL, and APRIL reporter cells. Unexpectedly, we found, that in these various systems, APRIL had no effect on survival of CLL cells, and activation of NF- κ B was not enhanced upon APRIL stimulation. Moreover, APRIL stimulation did not affect CLL proliferation, neither as single stimulus nor in combination with known CLL proliferation stimuli. Lastly, the survival effect conveyed by macrophages to CLL cells was not affected by TACI-Fc, an APRIL decoy receptor. We conclude that the direct role ascribed to APRIL in CLL cell survival might be overestimated due to application of supraphysiological levels of recombinant APRIL.

INTRODUCTION

Interactions of chronic lymphocytic leukemia (CLL) cells with bystander cells in tumor microenvironments, such as the lymph node (LN), provide them with essential survival signals. Upregulation of pro-survival B-Cell Lymphoma 2 (BCL-2) family members occurs upon stimulation with T cells or with monocyte-derived cells such as macrophages or Nurse-like cells¹. While the Tumor Necrosis Factor (TNF) receptor ligand family member CD40L can account largely for the survival effect by T cells², several factors have been described to have a role in the CLL cell survival effect governed by monocyte-derived cells^{3,4}. A prominent factor in this context is the TNF family member A Proliferation-Inducing Ligand (APRIL)⁴.

Under physiological conditions, APRIL has diverse roles in the development of B cells. It binds to its cognate receptors Transmembrane Activator and CAML Interactor (TACI) and B-cell Maturation Antigen (BCMA) after which TNF Receptor Associated Factors (TRAFs) are recruited to the receptor that relay the signal intracellularly. APRIL has furthermore been shown to signal via binding to heparan sulphate proteoglycans on the cell surface of its target cell⁵. In healthy B cells, APRIL signaling has a role in the induction of CD40L-independent class-switch recombination⁶, proliferation⁷, and sustained survival of plasmablasts⁸. APRIL has been reported to be expressed by several cell types including macrophages⁹, stromal cells¹⁰, CLL cells¹¹, and Nurse-like cells¹², which are CLL cell-differentiated monocytes that have been shown to induce survival of CLL cells³. APRIL is produced as either a membrane bound or soluble factor, depending on which alternative transcript(s) is/are expressed by the cell¹³. Furthermore, APRIL can be synthesized as part of a hybrid transcript called TWEPRIL (TWEAK-APRIL) together with TNF-related weak inducer of apoptosis (TWEAK), after which it is anchored to the cell membrane by virtue of the TWEAK domain¹⁴. Both TWEPRIL and the secreted alpha transcript variant of APRIL can be cleaved by furin in the Golgi apparatus or at the cell membrane respectively¹⁵, while the membrane-bound delta variant lacks the furin cleavage domain¹³.

In its ability to support cells, APRIL contributes to the growth of several malignancies¹⁶ and serum APRIL levels are correlated with worse prognosis¹⁷, which was also shown for CLL¹⁸. Furthermore, APRIL overexpression by transgenesis in the T-Cell Leukemia/Lymphoma 1A (TCL1) CLL mouse model is associated with enhanced disease severity¹⁹ and APRIL transgenic mice show an enhanced proliferation of peritoneal B-1 cells²⁰, which are considered to be the precursor cells for CLL in mice²¹. These effects are thought to result from induction of CLL cell survival by APRIL via activation of Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells (NF- κ B)^{4,12}. Altogether, these data suggest a role for APRIL in CLL biology. These findings have however been questioned by other reports, in which no survival effect on CLL cells was found^{22,23}.

In order to mechanistically dissect the role of APRIL, we used several complementary approaches to study its effects on CLL survival, activation, proliferation, and to investigate its role in macrophage-mediated survival. Surprisingly, we could not detect a direct effect of APRIL on CLL cells. Furthermore, although macrophages induce CLL survival, this effect appears to be independent of APRIL.

RESULTS

APRIL is expressed by macrophages in the CLL LN and CLL cells express APRIL receptors

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We first addressed whether APRIL is expressed in the CLL LN by performing qPCR on total RNA lysates from CLL LNs. These results show that APRIL expression in CLL LNs was approximately 4 times higher compared to a control systemic lupus erythematosus (SLE) LN extract. As negative control, NIH-3T3 mouse embryofibroblasts (3T3) had no APRIL expression (Figure 1a).

Next, we verified this finding on protein level using immunohistochemistry by staining for APRIL and macrophage marker CD68. As APRIL has been described to induce cell proliferation⁷, we also stained for proliferation marker Ki67. APRIL was expressed by the large majority of CD68⁺ cells in both CLL and SLE LNs, but there was no spatial association with Ki67⁺ lymphocytes in the CLL LNs (CLL LN Figure 1b and SLE LN Figure S1). Furthermore, expression of APRIL receptors BCMA and TACI was clearly detectable on CLL cells isolated from peripheral blood (PB) (Figure 1c).

In summary, APRIL is expressed in the CLL LN by macrophages and APRIL receptors are present on CLL cells.

No survival effect on CLL cells by *in vitro* APRIL stimulation

To explore direct functional effects of APRIL on CLL cells, we transduced NIH-3T3 cells with 3 different membrane-docked APRIL constructs (Figure 2a). We thus generated a system similar to the widely used TNF-family member CD40L overexpressing NIH-3T3 line (3T40)²⁴⁻²⁶, thereby ensuring trimerization of APRIL and expression on the cell membrane. The first cell line expresses the membrane bound TWEPRIL hybrid mRNA, with mutated furin consensus sites to render it uncleavable (3TA). In the second and third constructs (3T4A and 3T4sA), the intracellular and transmembrane regions of CD40L were fused to the extracellular domain of APRIL, without or with an interposed spacer ("s") region. The 3T40 cell line²⁴⁻²⁶ was used as a control.

APRIL expression in these cell lines was then verified by qPCR (Figure 2b) and western blot (Figure 2c) and signaling competence was tested using Jurkat-TACI:FAS (JTF) reporter cells²⁷ (Figure 2d). These JTF cells undergo apoptosis upon TACI signaling as a result of intracellular FAS domains, and provide a sensitive readout for APRIL binding to its cognate receptor (Figure 2a). Conditioned medium from APRIL overexpressing HEK293T cells (rhA med) and recombinant human APRIL (data not shown) were included as controls (Figure 2d). These data showed that all cell lines from our *in vitro* co-culture system express APRIL and that the expressed APRIL is able to signal via TACI.

These APRIL expressing 3T3 cells were subsequently used to test whether APRIL induced CLL cell survival. In contrast to 3T40 cells, we found no survival effect by any of the APRIL constructs or by recombinant human APRIL (rhA) after 72h co-culture (Figure 2e). Similarly, we could not detect a survival effect of conditioned supernatant from APRIL transfected HEK293T cells compared to supernatant from mock transfected cells (data not shown and Figure S2). Using the same APRIL stimuli, survival of CLL cells was measured at later time points (3, 6 and 10 days). In accordance with the results obtained at T=72h,

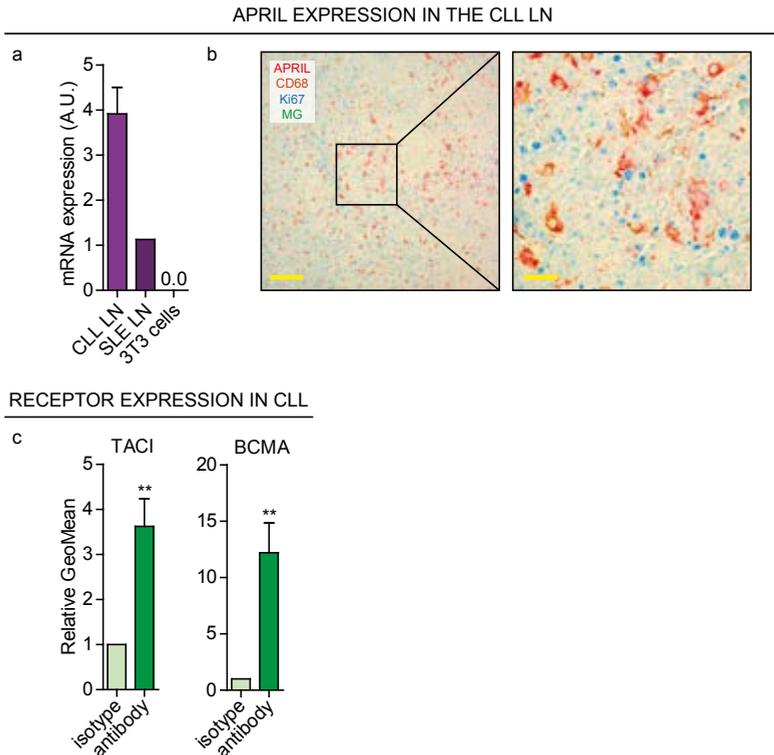


Figure 1: APRIL is present in the CLL LN and CLL cells express APRIL receptors. **a.** After total RNA lysis of paraffin embedded LN material or control NIH-3T3 (3T3) cells, APRIL mRNA levels were determined by performing a qPCR on CLL LN material (N=3) and an SLE LN as positive and 3T3 cells as negative control. All qPCRs were performed in triplo. A.U. denotes arbitrary units. **b.** Paraffin embedded LN slides from 6 CLL patients were immunohistochemically stained for APRIL, macrophage marker CD68, proliferation marker Ki67 and nuclear counterstain Methyl Green (MG). Data shown is representative of N=6. Scale bar represents 200 μ m (left) or 50 μ m (right). **c.** CLL cells (N=6) isolated from PB were stained for APRIL receptors TACI and BCMA or with the relevant isotype controls and analyzed by flow cytometry. Bars show mean \pm s.e.m.**, P<0.01 in a paired T-test.

APRIL did not significantly increase CLL survival, although a minor effect could be observed at day10 for rhA (Figure S2), as reported before²⁸.

No NF- κ B activation, activation marker expression or cell division in CLL cells exposed to APRIL

As several TNF family members can induce NF- κ B²⁹ we investigated if APRIL is able to induce NF- κ B activation by performing an NF- κ B DNA binding ELISA and found that, as expected, 3T40 cells induced both the canonical (p65) and non-canonical (p52) pathway in CLL cells. In contrast, no NF- κ B activation could be detected after stimulation with various APRIL constructs or rhA (Figure 3a), and known NF- κ B target transcripts were not induced (data not shown).

IN VITRO APRIL SYSTEM

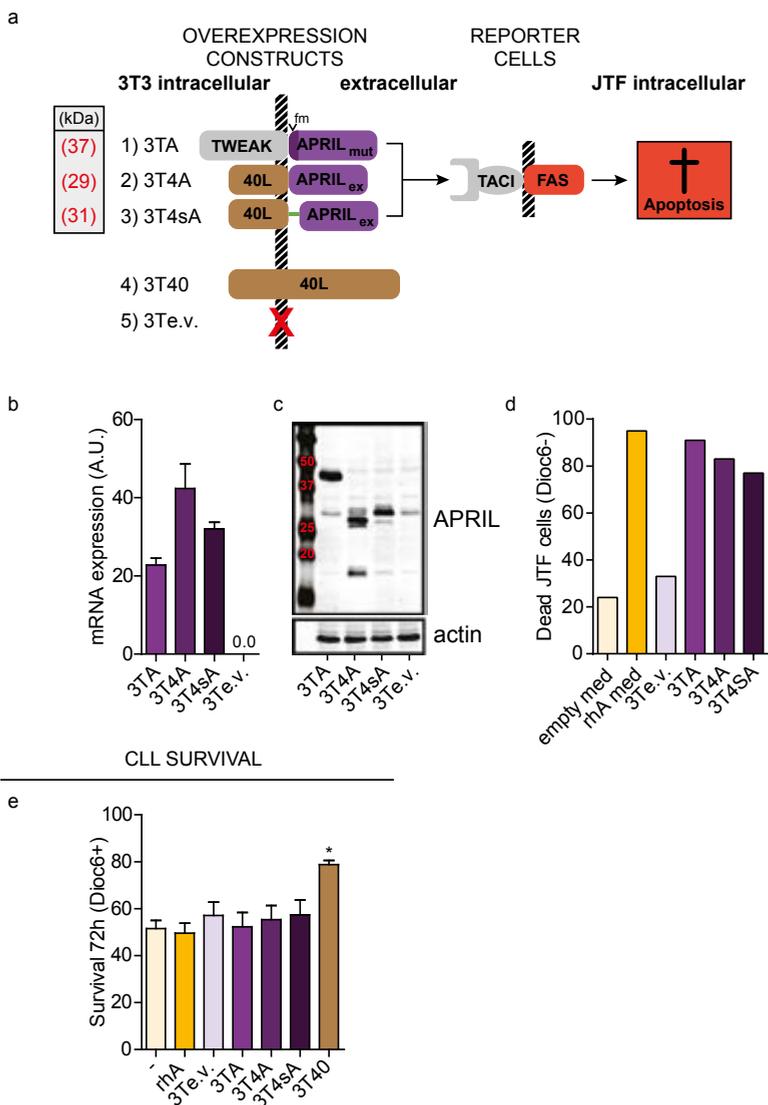


Figure 2: APRIL does not induce CLL cell survival. **a.** Depiction of APRIL overexpressing cell lines, control cell lines, and reporter cells used in co-culture experiments. NIH-3T3 cell lines overexpressing three different membrane-bound APRIL constructs were created (see methods). Apoptosis in APRIL reporter JTF cells is induced upon APRIL signaling, as TACI signaling triggers the FAS cell-death pathway. Full-length CD40L overexpressing 3T3 cells (3T40) and empty-vector transduced 3T3 cells (3Te.v.) are used as controls. Mutated furin sites are indicated by "fm", the spacer region is depicted by a green line. All constructs are drawn to scale. **b.** APRIL mRNA expression levels of the different APRIL overexpressing cell lines were tested by qPCR and compared to cells overexpressing empty pBabe vector (3Te.v.). The qPCR was performed in triplo and bars show mean \pm s.e.m., A.U. denotes arbitrary units. **c.** APRIL protein expression levels of the different APRIL overexpressing cell lines were tested by western blot and compared to cells overexpressing empty pBabe vector (3Te.v.). The predicted molecular weights of the APRIL fusion proteins are indicated in Figure 2a. **d.** Cell

Strong CD40 stimulation via cell-bound CD40L induces high-level NF- κ B activation in CLL cells. We have previously found that a CD40 stimulating antibody that induces moderate stimulation is able to upregulate activation markers CD58, CD80 and also CD95 (data not shown), indicating a higher sensitivity of this read-out. We therefore tested the potential of APRIL in this context, but in contrast to CD40L stimulation, APRIL stimulations did not upregulate the indicated markers (Figure 3b).

To study APRIL's potential involvement in CLL cell proliferation¹⁶, cell division was traced using Carboxyfluorescein succinimidyl ester (CFSE) labeling and the division index was calculated after various proliferation stimuli in the presence or absence of rhA. In line with a previous report², we found an increased proliferation of CLL cells after stimulation with CpG + IL2, 3T40 + CpG, and 3T40 + IL21, but no effect of rhA either as a single agent or in combination with these stimuli (Figure 3c).

Summarizing, while we found that CD40L is able to induce NF- κ B activation in CLL cells, activation marker expression and cell proliferation in combination with CpG or IL21, similar effects were not detectable after APRIL stimulation.

Macrophage-mediated CLL survival is independent of APRIL

We³⁰ and others³ have previously found that monocyte-derived cells such as macrophages are able to induce survival of CLL cells, and it was suggested that survival by monocyte-derived cells is dependent on APRIL¹². Although we did not observe a survival effect of stimulation with APRIL as a single stimulus (Figure 2e), the effects of APRIL could be dependent on other macrophage-expressed cytokines.

We therefore first generated M1 macrophages *in vitro* by differentiating healthy donor-isolated monocytes with IFN- γ . We then tested whether APRIL was expressed by these macrophages on western blot and found high expression in differentiated macrophages compared to low expression in monocytes and no expression in control 3T3 cells (Figure 4a inset and Figure S3). The APRIL signaling capacity of these macrophages was then tested by comparing cell death induced by macrophages in JTF reporter cells to the JTF death-to-rhA dose-response curve. The APRIL signaling capacity of macrophages was between that of 0 and 3.13 ng/mL rhA (Figure 4a).

To inhibit potential APRIL signaling during macrophage stimulation, we used TACI-Fc, a chimeric decoy receptor for APRIL³¹. We tested the activity of TACI-Fc by its ability to inhibit macrophage-induced cell death of JTF reporter cells cultured on

- ▶ lines described in Figure 2a were seeded as feeder layers and JTF reporter cells²⁷ were plated on top. Concurrently, JTF reporter cells were cultured in conditioned medium from APRIL (rhA med) or mock (empty med) transfected HEK293T cells. After 24h co-culture, the percentage of dead (Dioc6 negative) JTF reporter cells was determined by Dioc6-PI staining. **e.** CLL cells were cultured for 72h without stimulation (-) or with 200 ng/mL recombinant human APRIL (rhA). Likewise, CLL cells were co-cultured on the indicated APRIL expressing or control cell lines. Next, survival was determined by Dioc6-PI staining. Viable cells were defined as Dioc6 positive cells. CD40L overexpressing feeder cells (3T40) were used as a positive control for CLL cell survival. Bars show mean \pm s.e.m. for N \geq 8 for each condition. *, P<0.05 in a one-way analysis of variance (ANOVA) with Tukey post-hoc tests. When testing for significant differences, rhA was compared to unstimulated cells and 3T3 overexpression cell lines to 3Te.v.

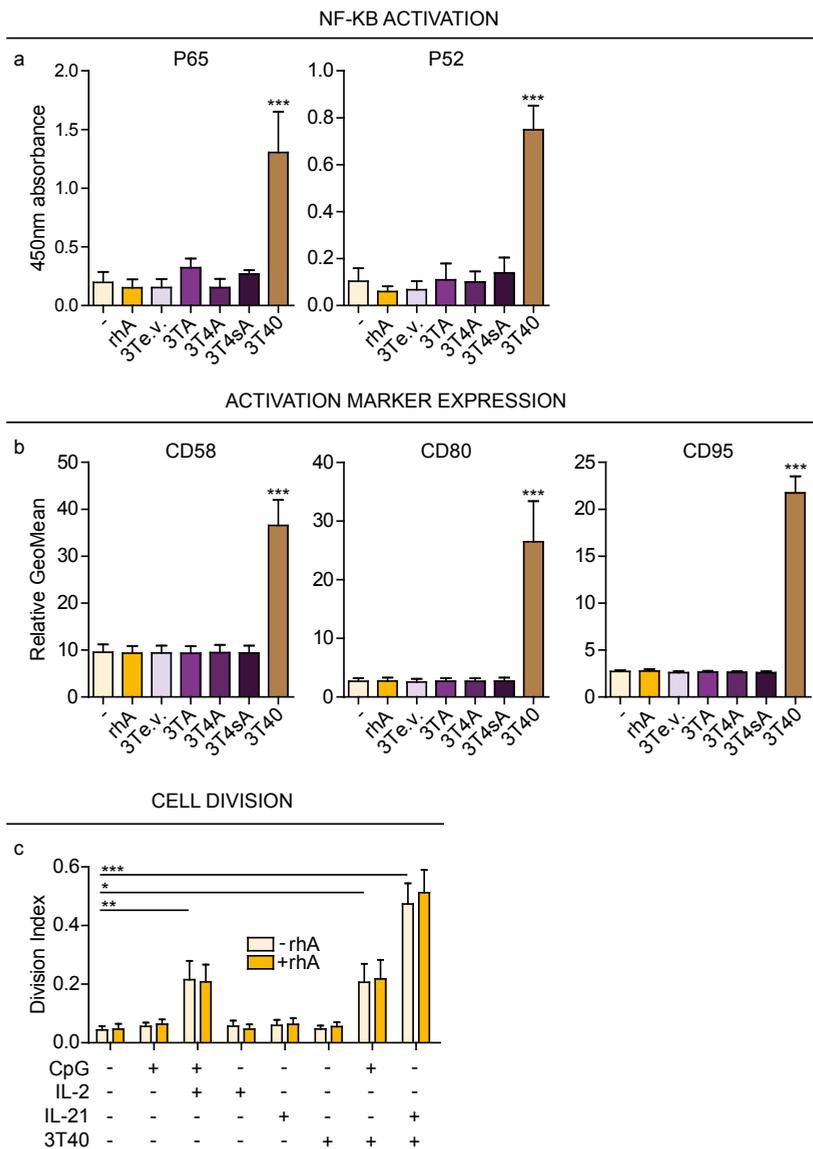


Figure 3: APRIL does not induce NF-κB signaling, activation marker expression, or cell division in CLL cells. **a.** CLL cells were cultured as in Figure 2e and nuclear extracts were prepared after 24h. The binding of activated canonical p65 and non-canonical p52 NF-κB subunits to consensus sequence oligonucleotides was then determined using ELISA. CD40L overexpressing feeder cells (3T40) were used as a positive control for NF-κB activation. Bars show mean \pm s.e.m. for N=3 for 3TA and 3T4sA and N=5 for the other conditions. ***, P<0.001 in an ANOVA test with Tukey post-hoc analysis. When testing for significant differences, rhA was compared to unstimulated cells and 3T3 overexpression cell lines to 3Te.v. **b.** CLL cells were cultured as in Figure 2e for 72h and expression levels of activation markers CD58, CD80 and of CD95 were determined using flow cytometry. CD40L overexpressing feeder cells (3T40) were used as a positive control for activation marker induction. Bars show mean \pm s.e.m. for N=3. ***, P<0.001 in an ANOVA test for repeated measures with Tukey

macrophages. We found that TACI-Fc dose-dependently reduced APRIL signaling from macrophages (Figure 4b).

We then cultured CLL cells on macrophages and measured CLL survival in the absence or presence of 2.5 $\mu\text{g}/\text{mL}$ TACI-Fc, the concentration at which macrophage-induced APRIL signaling was completely reverted. These data show that abrogation of APRIL signaling did not decrease the survival effect conveyed by macrophages (Figure 4c), suggesting no direct role for APRIL in macrophage-mediated CLL survival. Similarly, when culturing CLL cells on Nurse-like-cells¹² generated by 10 days stimulation of monocytes with CLL cells, inhibition of APRIL signaling by TACI-Fc did not affect CLL survival (Figure 4d).

DISCUSSION

We studied potential effects of TNF-family member APRIL on CLL cells, using complementary approaches and Jurkat-TACI:FAS reporter cells to verify the functionality of recombinantly expressed APRIL and the TACI-Fc decoy receptor. In contrast to our initial expectations, we could not detect an effect of APRIL on either CLL cell survival, cell activation, NF- κ B activation or cell proliferation. In addition, we could not detect a direct role of APRIL in macrophage-mediated CLL cell survival.

Various studies reported on the effects of APRIL on CLL. While some publications show an increased *in vitro* survival of CLL cells by rhA when used at a concentration of 500ng/mL^{4,12}, our experiments using 200ng/mL rhA (Figure 2e) are in line with the data of several other groups that were unable to find effects of recombinant APRIL, either alone²² or in combination with BAFF and CXCL12²³. Also, we established that the amount of APRIL produced by macrophages is >100 orders of magnitude lower compared to concentrations used in the reports that detect survival by APRIL. While APRIL may induce survival at high concentrations^{4,12}, this effect might be supraphysiological. Furthermore, concerning the survival effect of APRIL on non-malignant B cells, several groups have shown that APRIL is also dispensable in this context³².

In the TCL1 mouse model for CLL, we found that overexpression of human APRIL results in enhanced disease progression and shorter survival¹⁹. In light of these results, our current *in vitro* findings were also unexpected. In the APRIL overexpressing TCL1 model, the construct encoding human APRIL is under control of the Lck promoter. APRIL is thus predominantly expressed by T cells, and is present in the serum at a concentration comparable to our *in vitro* systems (data not shown). As T cells interact with other lymphoid cells including (leukemic) B cells but also with myeloid-derived immune cells it

- post-hoc analysis. When testing for significant differences, rhA was compared to unstimulated cells and 3T3 overexpression cell lines to 3Te.v. **c.** CFSE-stained CLL cells were cultured with various stimulations as indicated and as described², each time with or without rhA. After 4 days, the CFSE dilution was visualized by flow cytometry and division indices were calculated. Bars show mean \pm s.e.m. for N=6. *, P<0.05; **, P<0.01; ***, P<0.001; Each stimulation without rhA (light colored bars) was compared to the unstimulated condition in an ANOVA test for repeated measures with Dunnett's post-hoc analysis and the differences between - and + rhA for each condition were determined using a two-way ANOVA with Bonferroni correction.

cannot be ruled out that the observed effects occur indirectly, via other cells in the tumor microenvironment. Theoretically, the differences could also be due to distinct APRIL effects in the mouse compared to human situation.

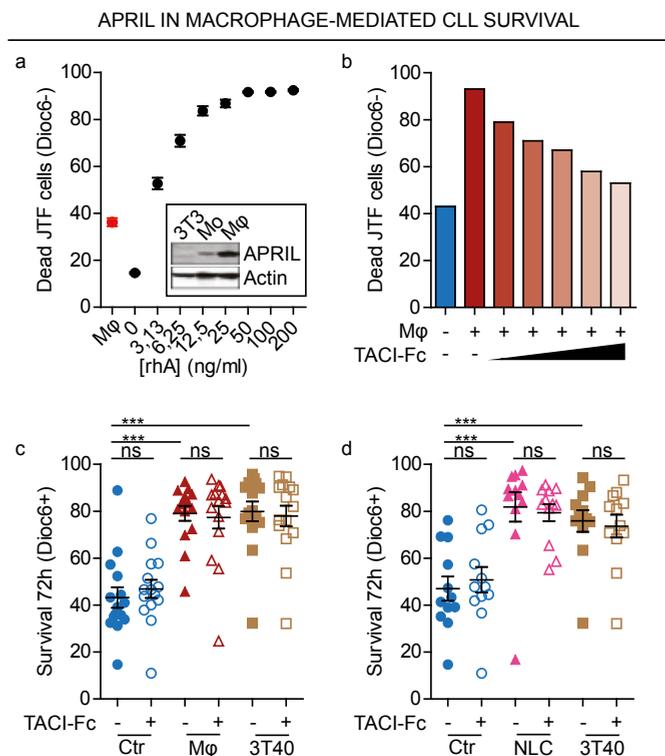


Figure 4: APRIL is expressed by macrophages, but has no role in macrophage-mediated survival. **a.** JTF reporter cells were stimulated for 24h with different concentrations of rhA or with M1-differentiated macrophages. Consequently, cell viability was determined as in Figure 2d and the macrophage-induced cell death was plotted alongside of the rhA titration curve. All conditions were performed in triplo and mean \pm s.e.m. are shown. Inset: APRIL expression was determined in these macrophages (M ϕ) by western blot and compared to monocytes (Mo) and untransduced 3T3 cells as negative control. **b.** JTF reporter cells were stimulated with M1-differentiated macrophages as in Figure 4a in the presence of increasing concentration of the APRIL decoy receptor TACI-Fc (from 0.25 μ g/mL to 2.5 μ g/mL) or control IgG after which cell viability of the JTF cells was measured as in Figure 2d. **c.** Confluent feeder layers of macrophages (M ϕ) were generated as in figure 4a and 3T40 feeder layers as in Figure 2e. These feeder layers or empty wells (Ctr) were then pre-incubated for 30min with TACI-Fc in order to suppress APRIL signaling or control IgG after which CLL cells were added on these feeder layers and co-cultured for 72h. Next, survival of the CLL cells was determined as in Figure 2e. Each point is one CLL sample (N=15) cultured in the indicated condition and mean \pm s.e.m. are indicated. ***, $P < 0.001$; ns, not significant, in an ANOVA test for repeated measures with Dunnett's post-hoc analysis. **d.** Confluent feeder layers of Nurse-like-cells (NLC) were generated by differentiating monocytes for 10 days using CLL cells. After washing, their survival effect on CLL cells in the presence of absence of TACI-Fc was determined as in Figure 4c. Each point is one CLL sample (N=12) cultured in the indicated condition and mean \pm s.e.m. are indicated. ***, $P < 0.001$; ns, not significant, in an ANOVA test for repeated measures with Dunnett's post-hoc analysis.

We found no evidence that CLL proliferation is enhanced either *in vitro* or *ex vivo* by APRIL. These data are in line with another publication in which no significant proliferative effect of APRIL medium in the presence of CpG was found²⁸. Studies on the effects of APRIL on proliferation of healthy B cells have been inconclusive; APRIL knockout mice for instance show normal B cell proliferation *in vitro*³³ and mice deficient for the APRIL receptor TACI paradoxically show increased B cell proliferation³⁴, whereas BCMA knockout mice show no overt phenotype³⁵.

In conclusion, our data indicate that APRIL does not directly mediate survival and proliferation of CLL cells. Consequently, APRIL signaling as therapeutic target in CLL might be beneficial in consideration that potential effects might be indirect.

MATERIAL AND METHODS

Patient samples

Patient material was obtained from CLL patients, after written informed consent and approval by our Ethical Review Board in agreement with the Helsinki Declaration of 1975, revised in 1983, as described before³⁶. All samples contained at least 90% CD5⁺/CD19⁺ cells (Supplemental table 1). In all experiments, CLL cells were used at a final concentration of 1.5×10^6 cells/mL.

APRIL overexpression cell lines and other APRIL stimulations

Mouse embryofibroblasts NIH-3T3 cells (DSMZ, Braunschweig, Germany), were transduced for stable overexpression with pBABE vectors expressing 1) TWEPRIL (NM_172089.3) with mutated furin cleavage sites (92RR→AA and 104RR→AA), 2) the transmembrane domain of CD40L (amino acids 1-112 of NM_000074.2) fused to the extracellular part of APRIL (amino acids 105-252 of NM_003808.3) without a linker region, or 3) with a linker region (PAAAAAASAAAAAAWVPVAT) (Figure 2a), 4) CD40L³⁷ or 5) empty vector, and all transduced cells were selected using puromycin. All constructs were sequence verified before transduction. Cells were cultured in IMDM supplemented with 10% FBS, 100 u/mL Penicillin-100 µg/mL Streptomycin (Life Technologies, Austin, TX, USA), 2mM L-glutamine (Life Technologies) and 0.00036% β-mercaptoethanol (Sigma, St. Louis, MO, USA) (IMDM+/+). When used as adherent feeder layer, fibroblasts were irradiated (30Gy) to stop proliferation before being seeded. After feeder cell adhering, CLL cells were plated on the respective cells. Where indicated (rhA) 200 ng/mL recombinant human APRIL (Peprotech, Rocky Hill, NJ, USA) was added to the culture medium, or culture medium conditioned on rhA-overexpressing HEK293T cells was added to the CLL cells at 80% final volume (rhA med).

Immunohistochemistry

Paraffin embedded CLL LN tissue was obtained from our institute's pathology department. Four-micron sections were de-waxed by immersion in xylene and hydrated by serial immersion in ethanol and TBS. Antigen retrieval was performed by heating sections for 20 min in sodium citrate buffer (10mM sodium citrate, 0.05% Tween20, pH 6.0). Sections were washed with TBS (2 x 10 min) and blocked with Ultra V block (Thermo

Scientific, Waltham, MA, USA) for 10 min at T_{room} . Sections were incubated with primary antibody APRIL-y2 (1:1000 Enzo LifeSciences, Farmingdale, NY, USA) in normal antibody diluent (ImmunoLogic, Duiven, The Netherlands) O/N at 4°C. After washing, sections were incubated with post-antibody block (ImmunoLogic) and subsequently incubated with secondary Polymer a-Rb/AP antibody (ImmunoLogic) followed by visualization by Vector Red (Vector Laboratories, Burlingame, CA, USA). A second antigen retrieval was performed for 10 min at 98°C in TRIS-EDTA (pH=9.0) and after Ultra V block, sections were incubated with a combination of primary antibodies directed against CD68 (PG-M1, Dako, Carpinteria, CA) and Ki67 (SP6, Clinipath, Duiven, The Netherlands), both 1:2000 in normal antibody diluent for 1h at T_{room} . After washing, a combination of Polymer a-Rb/AP and Polymer a-Ms/HRP (both Immunologic), was added for 30 min at T_{room} and antibody binding was visualized by Vector Blue (Vector Laboratories) and subsequent DAB (ImmunoLogic) staining after which slides were counterstained with methyl green and mounted with vectamount. Slides were visualized using a Leica DMLB microscope (Leica Microsystems, Buffalo Grove, IL, USA) equipped with a Leica DFC420 camera and cropped using Adobe Illustrator CS5 software (Adobe, San Jose, CA, USA).

APRIL reporter cell assays

To measure APRIL signaling, Jurkat-TACI:FAS (JTF) reporter cells provided as a kind gift by P. Schneider²⁷ were cultured with different APRIL stimuli for 24h after which cell death of JTF cells was measured by Dioc6-PI staining as described before³⁶.

Flow Cytometry and cell viability

Cell viability was measured by Dioc6-PI staining as described before³⁶. Flow cytometrical staining for APRIL receptors was performed using the TACI-PE (BD Bioscience, San Jose, CA, USA) and BCMA-FITC (Enzo) antibodies as described previously² and stained cells were analyzed on a FACS Canto II (BD). Data was then analyzed using FlowJo 9.9 (FlowJo LLC, Ashland, OR, USA).

Western blot

Western blotting was performed as described previously³⁸, using the α -human APRIL-y1 antibody (Abcam, Cambridge, MA, USA) and β -actin (Santa Cruz, Dallas, TX, USA) as a loading control. IRDye 680 donkey anti-rabbit IgG and IRDye 800 donkey anti-goat IgG (Westburg, Leusden, The Netherlands) were used as secondary antibodies.

Real-time polymerase chain reaction (qPCR)

Total RNA was isolated from paraffin embedded CLL LN material or from APRIL overexpressing 3T3 cells using the GeneElute™ Mammalian Total RNA Miniprep kit (Sigma) and cDNA was created by reverse transcriptase reaction according to manufacturer's instructions (Promega, Madison, USA). APRIL and household gene hypoxanthine phosphoribosyltransferase 1 (HPRT) were amplified using exon-exon boundary overlapping probes (APRIL CTGCTATAGCGCAGGTGTCTT and GGAAGGTTCCATGTGGAGAG; HPRT CCTGGCGTCGTGATTAGTGA and CGAGCAAGACGTTTCAGTCCT) in a SYBR green (Life

Technologies, Austin, TX, USA) reaction (40 cycles of 3 sec at 95°C followed by 30 sec at 60°C). The expression of APRIL was then calculated per sample as the difference in Ct values between the APRIL signal and HPRT signal using the formula $1000 \times 2^{-(Ct_{APRIL} - Ct_{HPRT})}$.

Cell proliferation assays

Cell proliferation was assessed by Carboxyfluorescein succinimidyl ester (CFSE) cell tracing as described before², using 200 ng/mL rhAPRIL (Peprotech) and other reagents as described before². Division indices were calculated using FlowJo 9.9 (FlowJo LLC).

Macrophage and Nurse-like-cell experiments

Monocyte-derived macrophages and Nurse-like cells were obtained by differentiating monocytes isolated from healthy donor buffy coats obtained from the central blood bank after obtaining written informed consent. To this end, PBMCs were isolated using ficoll gradient purification (Lucron, Dieren, The Netherlands), after which monocytes were separated from peripheral blood lymphocytes using percoll gradient purification (GE healthcare, Milwaukee, USA), both according to manufacturer's instructions. Next, monocytes were incubated to adhere at 37°C in 5% CO₂ for 40 min at a concentration of 0.75*10⁶ cells/mL in 6-well plates (3mL) in IMDM/1% Fetal Bovine Serum (FBS, Invitrogen, Carlsbad, CA, USA) and washed to remove non-adherent cells. The monocytes were then differentiated towards M1 macrophages using 10ng/mL IFN-γ (R&D systems, Minneapolis, MN, USA) in IMDM+/+ for 72h or to Nurse-like-cells by differentiating them using CLL cells for 10 days³. After washing the macrophages or Nurse-like cells twice, they were pre-incubated for 30 min with TACI-Fc (R&D systems) or an equimolar concentration of control IgG (R&D systems) to obtain a final concentration of 2.5 μg/mL of TACI-Fc. Next, thawed CLL cells were added. After 72h, cell viability was measured as described before³⁶.

Statistical analysis

One-way analysis of variance (ANOVA) with Tukey post-hoc tests (comparing all groups to each other) or Dunnet's post-hoc test (comparing all groups to one group) were performed to test for significant differences between multiple groups. When applicable, tests were adjusted for repeated measures. A two-way ANOVA with Bonferroni post-hoc tests was used when testing for differences between groups with two independent variables. When testing for differences between two groups, a T-test was used. P values <.05 (*), <.01 (**) and <.001 (***) were considered statistically significant, non-significance is not indicated except in Figure 4c and 4d.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIALS

3

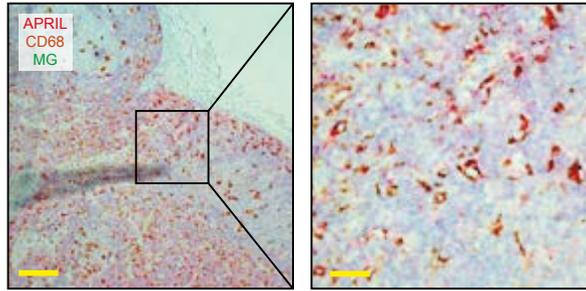


Figure S1: CD68-APRIL staining in SLE LN. A paraffin embedded LN slide from an SLE patient was immunohistochemically stained for APRIL, macrophage marker CD68, and nuclear counterstain Methyl Green (MG). Scale bar represents 200µm (left) or 50µm (right).

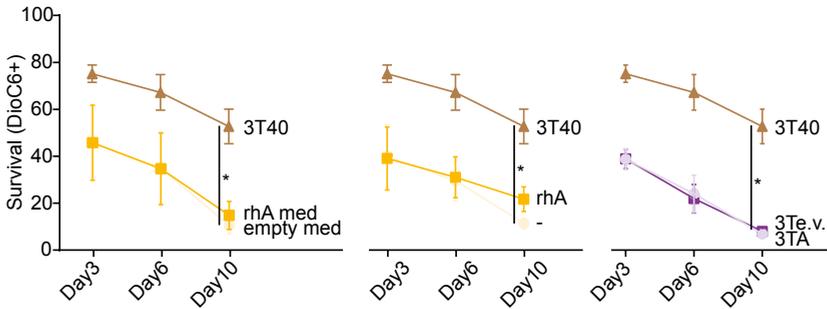


Figure S2: Long term survival of CLL cells using different APRIL stimuli. CLL cells were cultured with the APRIL stimulations used in Figure 2d and Figure 2e and survival was measured at indicated time points. All CLL samples were pre-incubated O/N with 1.5 µg/mL cytosine guanine dinucleotide to induce TACI and BCMA upregulation²⁸. Points show mean \pm s.e.m. for N=3 patients for each condition. *, $P < 0.05$ in a paired T-test comparing stimulated conditions (APRIL stimulations or 3T40) with the respective control conditions (-, empty med, 3Te.v.). Only significantly different data points at day 10 are indicated for readability.

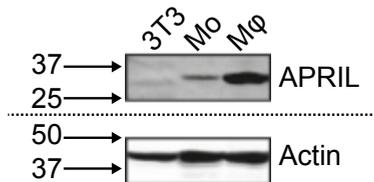


Figure S3: Size markers for western blot of Figure 4a. Size markers for the western blot of Figure 4a (inset) are indicated by arrows. The predicted mass for APRIL is 27kDa.

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4

THE APRIL PARADOX IN NORMAL VERSUS MALIGNANT B CELL BIOLOGY

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Chronic lymphocytic leukemia (CLL) is a classic example of a malignancy that engages in interactions with bystander cells such as T cells, stromal cells, and monocyte derived cells (MDCs) to provide itself with essential survival and proliferative signals. The important role of MDCs in the lymph node has recently been highlighted by the observation that their depletion in the T-cell leukemia/lymphoma protein 1A (TCL1) CLL mouse model by using clodronate-containing liposomes results in a better survival¹. Within the microenvironment, several factors can contribute to CLL protective effects, and among these a significant role has been attributed to Tumor Necrosis Factor (TNF)-family members CD40L, B-cell activating factor (BAFF) and A proliferation inducing ligand (APRIL)². The effects of T cell factor CD40L on both non-malignant and CLL B cells are well established: CD40L has been shown to activate both non-malignant and CLL B cells via NF- κ B activation, thereby inducing a survival advantage via upregulation of several BCL-2 family members. CD40L can furthermore lead to B cell receptor-independent proliferation in both malignant and non-malignant cells, and induces class-switch recombination in non-malignant B cells (reviewed by Elgueta et al.³).

APRIL and BAFF are produced by MDCs and can bind to their cognate receptors Transmembrane activator and CAML interactor (TACI) and B-cell maturation antigen (BCMA) on target cells. In addition, BAFF can bind to a third receptor called BAFF-R². In both B cell physiology and pathology, APRIL and BAFF have been ascribed roles equally important as CD40L: both APRIL and BAFF have been reported to be responsible for the maintenance of plasma cells^{4,5} and can induce class-switch recombination². BAFF in addition activates NF- κ B in healthy B cells, and is involved in mature B cell survival, whereas APRIL is not. Moreover, BAFF is critical for B cell maturation, as both BAFF and BAFF-R knockout mice lack mature B cells, an effect that is not observed in APRIL knockout mice (reviewed by Mackay and Schneider²).

In the context of CLL, we have previously found that overexpression of APRIL in the TCL1 mouse model accelerated disease progression⁶. Furthermore APRIL was present in CLL LNs as shown in initial experiments using quantitative PCR and immunohistochemistry. Moreover, serum APRIL levels correlate with worse prognosis in CLL patients, and these effects have been attributed to NF- κ B-mediated induction of CLL cell survival⁷. These observations suggest that APRIL has an important role in CLL cell survival, but confusingly other groups were unable to recapitulate the survival effect *in vitro* using recombinant APRIL^{8,9}. In view of this growing controversy, in a recent Cell Death Discovery report, we used several complementary approaches to study the role of APRIL in (MDC-mediated) CLL cell survival and proliferation¹⁰.

We applied a novel APRIL overexpressing system that mimics the widely applied system of CD40L stimulation, by overexpressing a fusion protein of extracellular APRIL with either the transmembrane portion of CD40L or its natural fusion partner TWEAK (forming TWE-PRIL) in NIH-3T3 cells. These systems were compared to the effects of soluble APRIL produced by HEK-293 cells or recombinantly. After verifying signaling capacity using APRIL reporter cells, we analyzed direct survival effects of APRIL on CLL cells. Although CLL cells expressed both APRIL receptors TACI and BCMA, surprisingly we found no survival induction. Secondly, inhibition of APRIL using a TACI decoy receptor did not reduce *in vitro* macrophage-mediated survival, although these macrophages do

express APRIL. We quantified their APRIL production capacity, and found it to be less than 3.13ng/ml. In line with these negative results, APRIL stimulation did not induce canonical or non-canonical NF- κ B signaling, nor enhanced proliferation of CLL cells either alone or in combination with other stimuli¹⁰.

These observations pose a seeming paradox to APRIL's reported roles in healthy B cells, but this might be solved by looking at the developmental stage of the B cell (see Figure 1). It has been published that APRIL is able to induce survival in plasma cells⁴, yet no survival effects was found for other developmental stages⁴. Similarly, APRIL contributes to naive B cell proliferation, but this was not found in other B cell maturation phases⁵. With respect to CLL cells, the effects of APRIL might overlap with its effects on precursor cell from which the CLL cell is derived. Depending on the IgV_H mutation status, these precursor cells have been described to be memory B cells or B1 cells¹¹. Within these precursor cells, no induction of survival was found⁴ and effects on proliferation, or NF- κ B activation effects have not been reported. Possibly, these precursor cells have activated a cellular program that lacks APRIL responsiveness. If true, it becomes quite plausible

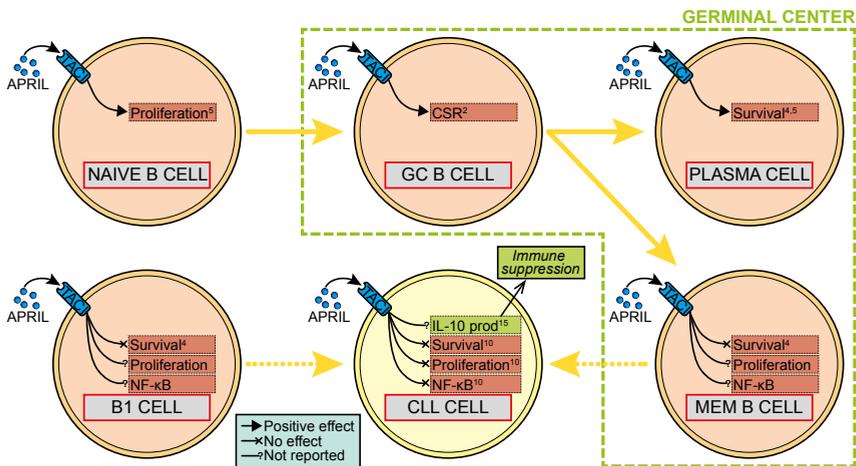


Figure 1: APRIL effects at different stages of B cell development and in CLL. Depending on the developmental stage of B cells, APRIL stimulation results in different outcomes. Whereas APRIL induces the survival of plasma cells for instance, it has no effect on survival in other stages. As CLL cells are derived from precursor B cells -namely memory B cells or B1 cells- that are unaffected by APRIL with respect to survival, proliferation or NF- κ B activation, the overlap in differentiation program could explain the absence of direct effects on CLL cells that we have reported in Cell Death and Discovery¹⁰. It can however not be excluded that APRIL exerts its effect on CLL cells indirectly via other cell types. Stimulation of TAC1 in B1 cells by BAFF has recently been shown to induce IL-10 production in these cells, that could result in immune-suppressive signaling, thereby mitigating cytotoxic T cell responses to the malignant cells. Numbers in boxes denote references. Arrows and lines denote positive effect, no effect or no reported effect of APRIL. Abbreviations: CSR, class-switch recombination; GC, germinal center; MEM, memory; prod, production

that CLL cells derived from these cells likewise are not affected by APRIL. In accordance with this line of reasoning, in multiple myeloma cells -that are derived from an APRIL dependent B cell stage- APRIL induces a strong survival effect¹².

The noted absence of NF- κ B activation after APRIL stimulation can be contrasted to the prominent role that BAFF plays in this light. This part of the puzzle can be explained by the fact that the non-canonical pathway can exclusively be activated via the BAFF-R², to which APRIL cannot bind. Although activation of TACI and BCMA can under certain circumstances result in canonical signaling¹³, this signaling might be dependent on the mode of activation of the receptor. Interestingly, as it has been shown that BAFF-mediated B cell survival is dependent on non-canonical signaling¹⁴, the lack of APRIL effects on CLL survival could be explained by these data.

Nevertheless, other reports⁷ do suggest a direct survival effect on CLL cells when using recombinant APRIL at a concentration of 500ng/ml. As pointed out above, the APRIL producing capacity of macrophages is apparently >100-fold lower than this. The observed effects using high APRIL concentrations might therefore be supra-physiological. Still, the enhanced disease progression observed in APRIL overexpressing TCL1 mice occurs at APRIL concentrations comparable to our human *in vitro* system, and clearly suggests that in the *in vivo* context APRIL contributes to CLL progression. A possible explanation for the apparent contrast between *in vivo* murine and *in vitro* human data might be that the role of APRIL in CLL pathogenesis might be indirect, via other cells. In a recent report, increased IL-10 production by regulatory B10 cells after stimulation of APRIL receptor TACI by recombinant BAFF was found¹⁵. This increased IL-10 production could in turn result in immune suppression, thereby contributing to the immune evasion of malignant CLL cells.

We propose that absence of direct APRIL effects on CLL cells conceivably reflects that they have switched on a cellular program that derives from their non-malignant precursor cells, that neither respond to APRIL. The effects of APRIL could however be mediated via other cells, such as IL-10 producing B10 cells, that act via indirect mechanisms on CLL cells.

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5

MACROPHAGES CONFER SURVIVAL SIGNALS VIA CCR1-DEPENDENT TRANSLATIONAL MCL-1 INDUCTION IN CHRONIC LYMPHOCYtic LEUKEMIA

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ABSTRACT

Protective interactions with bystander cells in micro-environmental niches such as lymph nodes (LNs) contribute to survival and therapy resistance of chronic lymphocytic leukemia (CLL) cells. This is caused by a shift in expression of BCL-2 family members. Pro-survival proteins BCL-X_L, BFL-1, and MCL-1 are upregulated by LN-residing T cells through CD40L interaction, presumably via NF- κ B signaling. Macrophages also reside in the LN, and are assumed to provide important supportive functions for CLL cells. However, if and how macrophages are able to induce survival is incompletely known.

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We first established that macrophages induced survival due to an exclusive upregulation of MCL-1. Next, we investigated the mechanism underlying MCL-1 induction by macrophages in comparison with CD40L. Genome-wide expression profiling of *in vitro* macrophage- and CD40L-stimulated CLL cells indicated activation of the PI3K-AKT-mTOR pathway, which was confirmed in *ex vivo* CLL LN material. Inhibition of PI3K-AKT-mTOR signaling abrogated MCL-1 upregulation and survival by macrophages as well as CD40 stimulation. MCL-1 can be regulated at multiple levels, and we established that AKT leads to increased MCL-1 translation, but does not affect MCL-1 transcription or protein stabilization. Furthermore, among macrophage-secreted factors that could activate AKT, we found that induction of MCL-1 and survival critically depended on C-C Motif Chemokine Receptor-1 (CCR1).

In conclusion, this study indicates that two distinct micro-environmental factors, CD40L and macrophages, signal via CCR1 to induce AKT activation resulting in translational stabilization of MCL-1, and hence can contribute to CLL cell survival.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is characterized by accumulation of monoclonal B cells in peripheral blood, lymph nodes (LNs) and the bone marrow. Interactions with bystander cells such as stromal cells, T cells or macrophages (Mφs) in the LN provide CLL cells with a survival benefit and resistance to chemotherapy, due to changes in the apoptotic balance in CLL cells¹. The important role of Mφs was very recently shown in Mφ depletion experiments in the TCL1 CLL mouse model, in which a better overall survival was observed².

With respect to relevant survival factors, we have previously shown that the effects of LN-residing T cells on CLL cells are largely governed by CD40L interaction, as CLL cells stimulated by CD40L and T cells have similar gene expression and apoptotic profiles³. Factors from monocyte-derived nurse-like cells that have been described to induce survival include CXCL12⁴, APRIL, and BAFF. These latter two factors are reported to induce NF-κB activation⁵. Using several complementary approaches, we however found negligible effects of APRIL in Mφ-mediated survival⁶, implying that other Mφ-factors must be involved.

Concerning the change in apoptotic balance, our group and others have previously shown increased expression of pro-survival B-cell lymphoma 2 (BCL-2) family members in CLL cells isolated from LNs⁷ as well as in CLL cells stimulated with T cell factor CD40L^{3,8-10}. Clinically, such changes in apoptosis regulation correlate with worse prognosis and resistance to chemotherapy, as several groups have shown for pro-survival proteins BCL2-related protein A1 (BFL-1) and B-cell lymphoma-extra large (BCL-X_L)^{11,12}, as well as Induced myeloid leukemia cell differentiation protein (MCL-1) levels¹³⁻¹⁶. The effects of monocyte-derived cells such as Mφs on the apoptotic balance are less well studied.

The negative prognostic impact of Mφs in CLL² and the fact that their extracellular and intracellular signaling events towards CLL cells are unknown, suggest that unraveling these pathways can contribute to development of new therapies. We therefore studied the effects of both Mφs and CD40L on CLL cell survival and identified chemokine receptor CCR1 as an important mediator of Mφ-induced CLL cell survival. Secondly, we found that within the CLL cell, both Mφs and CD40L increase AKT-mTOR dependent translation of MCL-1 protein.

RESULTS

T cells and Mφs induce CLL survival by changing the apoptotic balance

As we have shown previously that stimulation of CLL cells via CD40 almost fully mimics the effects of activated T cells on CLL³, we used NIH-3T3 cells transfected with CD40L (3T40 cells) as a model for the interaction with T cells. We also generated M1 and M2 differentiated macrophages (Mφs) from monocytes isolated from healthy donors by differentiation with IFN-γ (M1) or IL-4 (M2). Both types of Mφs and 3T40 cells increased survival of CLL cells after 72h co-culture (Figure 1a).

We analyzed 72h stimulated CLL cells for anti-apoptotic proteins MCL-1, BCL-X_L, BFL-1 and BCL-2 using western blot. MCL-1 was upregulated by both Mφ types and 3T40 stimulation, while BCL-X_L and BFL-1 were only upregulated by 3T40 stimulation, consistent with activation of NF-κB by 3T40 cells¹⁰ (Figure 1b, Figure S1 for size markers).

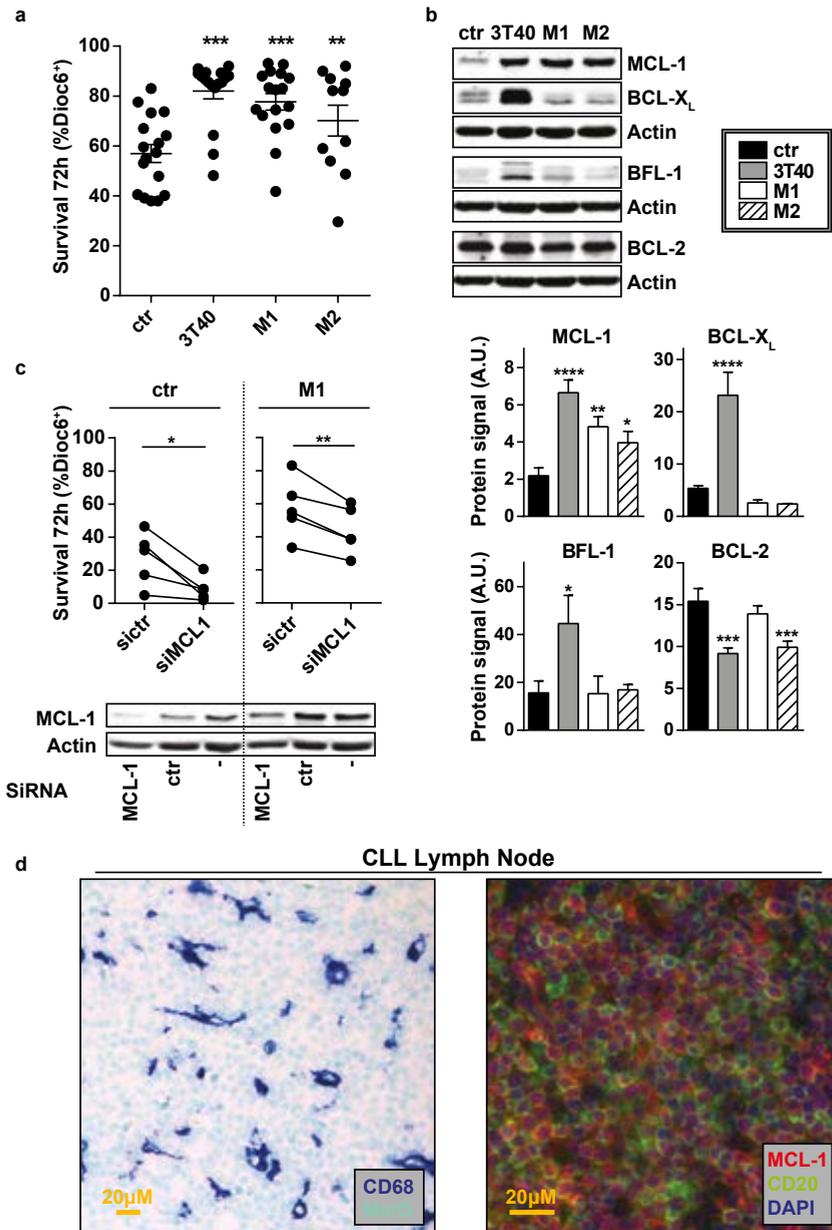


Figure 1: Mφs and CD40L induce CLL survival by changing the apoptotic balance. Confluent feeder layers of macrophages (Mφ) and non-dividing CD40L-overexpressing fibroblasts (3T40) were generated as described under methods. **a.** CLL cells were co-cultured on the indicated feeder layers or without feeder layer (ctr) for 72h after which survival was measured by Dioc6-PI staining. The percentage of viable cells was defined as Dioc6 positive cells. Each point represents one stimulation of a CLL sample. Shown are mean \pm s.e.m. **b.** After 72h co-culture as in Figure 1a, protein lysates of CLL cells were probed by western blot for the levels of pro-survival BCL-2 family members MCL-1, BCL-X_L, BFL-1 and BCL-2. β -actin was used as a loading control. Data shown is representative of at least N=6. To improve clarity, western blot images were cropped in this and following figures (upper panel). The levels of each

To verify the relevance of MCL-1 upregulation in the observed survival effect of M ϕ , siRNA interference using Amaxa nucleofection was applied, prior to co-culture on M1 M ϕ s (Figure 1c). The M ϕ -mediated survival effect was largely reverted after MCL-1 knockdown. Thus, in the M ϕ setting, CLL cells depend on the upregulation of MCL-1 for their survival.

The expression of MCL-1 in CLL LNs and the presence of M ϕ s were verified by immunohistochemical staining. These stainings indicated that MCL-1 is present at significant levels in LN-residing CLL cells and that the LN is interspersed with M ϕ s. (Figure 1d, and Figure S2 for single channel images).

MCL-1 induction depends on PI3K-AKT-mTOR signaling after both CD40L and M ϕ stimulation

To identify which intracellular pathway was responsible for the observed MCL-1 upregulation, genome-wide expression profiling (using microarrays) of stimulated CLL cells was performed. A comparison of the log-fold changes of the expression levels of each gene in stimulated cells versus the control condition showed a large overlap between regulated genes in M1 and M2 stimulated samples (Figure 2a; $R^2=0.72$). When comparing either M1 or M2 to 3T40 stimulated samples, there was more discordance and only limited overlap ($R^2=0.03$ and 0.00 respectively).

As both CD40L, M1, and M2 stimulation induced MCL-1 (Figure 1b), we hypothesized that the same upstream regulator was responsible for its induction by all stimuli. Using Ingenuity Pathway Analysis, potential upstream regulators for each condition were determined and these upstream regulators were marked in a scatterplot (Figure 2b). Interferon (IFN) α and phosphoinositide 3-kinase (PI3K), the upstream regulator of the PI3K/V-Akt Murine Thymoma Viral Oncogene Homolog (AKT)/mammalian target of rapamycin (mTOR) pathway, were predicted to be activated in all three conditions. These data were confirmed in a CAMERA pathway analysis using the Molecular Signatures Database v5.1 (MSigDB, Broad institute) (data not shown). Of note, neither M1 nor M2 M ϕ s expressed CD40L as determined by flow cytometry (data not shown), indicating that another factor is responsible for the observed M ϕ effect. As M1 and M2-stimulated CLL cells had highly similar gene expression and functional profiles (Figure 1a-b and Figure 2a), we used the more tightly adherent M1 M ϕ s for further experiments (hereafter called M ϕ).

- protein were quantified for 6 patients using densitometry and calculated as the protein signal relative to the Actin loading control. (lower panel). A.U. denotes arbitrary units. **c.** CLL cells were transfected with one of two siRNAs directed against *MCL-1* (siMCL-1) or a control (sictr) or not transfected (-) before co-culture on M ϕ s or without feeder layer (ctr). After 72h, the viability of CLL cells was determined as in Figure 1a. Each line represents one patient sample. Transfection efficiency using a GFP control plasmid has previously been determined to be approximately 50% (data not shown). (upper panel). Protein lysates of CLL cells were probed for MCL-1 levels by western blot to verify the knockdown. β -actin was used as a loading control. Data shown is representative of N=5. (lower panel). **d.** Paraffin embedded lymph node slides from CLL patients were immunohistochemically stained for M ϕ marker CD68 and nuclear counterstain Methyl Green (left panel), or MCL-1, B cell marker CD20, and nuclear counterstain DAPI (right panel). Data shown is representative of N=6.

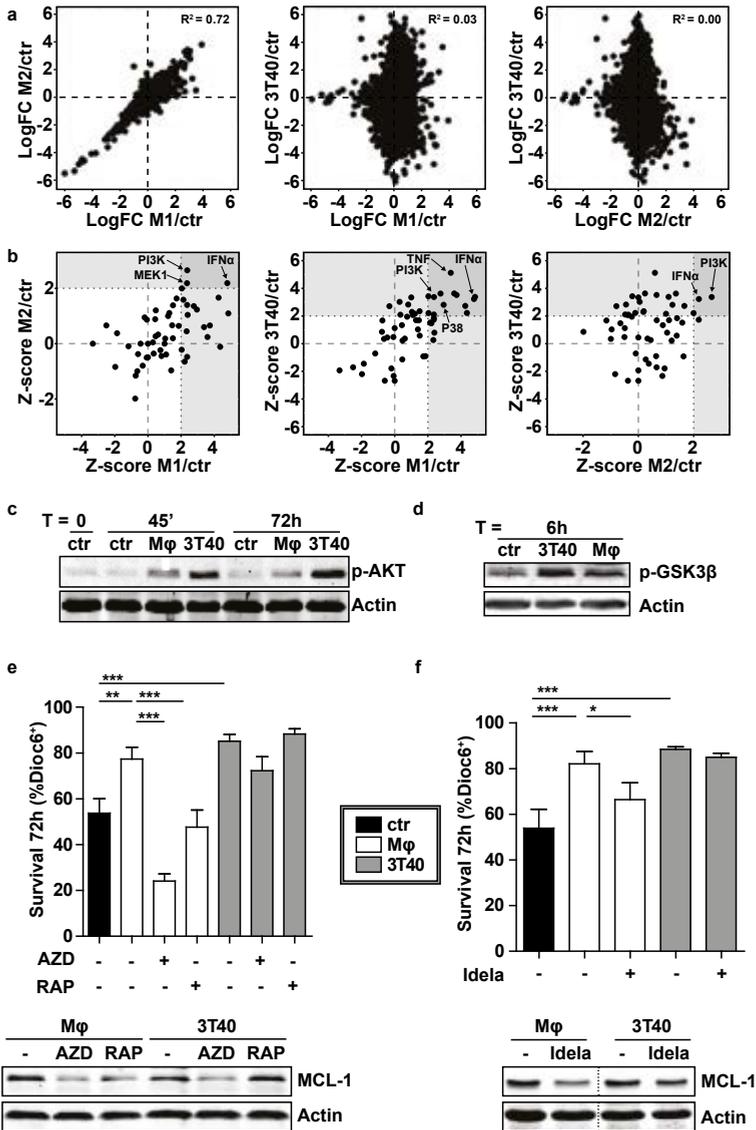


Figure 2: MCL-1 induction depends on PI3K-AKT-mTOR signaling after both CD40L and Mφ stimulation. **a.** CLL cells were co-cultured as in Figure 1 (N=3 paired samples for each condition) for 16h and RNA from CD5/CD19 FACS-sorted CLL cells (>99% purity) was subjected to microarray analysis. Next, for each mRNA, the log fold change (LogFC) relative to control condition was calculated and plotted. The goodness of fit for the linear regression models (R^2) is indicated. **b.** A core analysis in the Ingenuity Pathway Analysis (IPA) software was performed with signatures created from the microarray data based on cut-off values $\logFC > 0.8$ and P value < 0.01 to identify putative upstream regulators common to all three stimuli. The Z-score of candidate upstream regulators was visualized in scatterplots, in which upstream regulators with a Z-score over 2.0 were defined as activated, in line with IPA specifications. Several potentially relevant activated regulators are indicated by arrows. **c.** CLL cells were serum starved for 3h and subsequently co-cultured as in Figure 1 on indicated feeder layers for indicated time points and the phosphorylation of AKT was determined by western blot

Furthermore, considering that IFN α is not secreted by 3T40 cells, we decided to focus on the AKT pathway activation.

In accordance with the microarray data, we found that both 3T40 and M ϕ s induced AKT phosphorylation (Figure 2c). To test for sustained functional activation of AKT, phosphorylation of its downstream target GSK3 β ¹⁷ was probed after 6h, showing phosphorylation after both stimuli (Figure 2d). The phosphorylation of GSK3 β was furthermore found in FACS sorted CLL cells from LN material, indicating active AKT signaling *in vivo* (Figure S3).

To test whether MCL-1 induction depended on AKT-mTOR signaling, we used two pharmacological inhibitors of mTOR; AZD8055, which competes for the ATP binding pocket¹⁸ and the allosteric inhibitor rapamycin¹⁹. Both inhibitors were able to completely revert the CLL survival effect conferred by M ϕ s. However, they did not revert CD40-induced survival, likely due to the concurrent upregulation of anti-apoptotic proteins BFL-1 and BCL-X_L. AZD8055 reduced MCL-1 levels in both M ϕ and 3T40 stimulated CLL cells. Rapamycin on the other hand showed only an effect in CD40L-stimulated CLL cells (Figure 2e). Rapamycin, as opposed to AZD8055, is unable to inhibit the mTORC2 mediated positive feedback loop^{19,20} and mTOR mediated 4E-BP phosphorylation has been shown to be resistant to rapamycin treatment²⁰. Next, CAL101 (idelalisib), which inhibits PI3K δ , was used to decrease AKT activation²¹. Idelalisib treatment showed a reduction in MCL-1 protein for both conditions, but only reduced M ϕ - (and not 3T40-) mediated CLL survival, (Figure 2f).

Collectively, these data suggest that the observed induction of MCL-1 after CD40L or M ϕ stimulation depends on PI3K-AKT-mTOR signaling.

MCL-1 is induced on the translational level

Protein stabilization via AKT-dependent GSK3 β phosphorylation has been attributed an important role in MCL-1 induction (22-24). We therefore measured MCL-1 turnover after addition of translation inhibitor cycloheximide. However, these analyses indicated similar stability of MCL-1 protein before and after co-culture with M ϕ or 3T40 cells (Figure 3a). Furthermore, treatment with CHIR99021, a selective GSK3 inhibitor, induced no changes

- (shown blot is representative of N=4). β -actin was used as a loading control. **d.** After resting for 3h, CLL samples were co-cultured as in Figure 1 on indicated feeder layers for 6h and the activation of AKT signaling was determined by western blotting for phosphorylated GSK3 β (blot representative of N=4). **e.** CLL samples were co-cultured as in Figure 1 in the presence or absence of mTOR inhibitors AZD8055 (500nM) or rapamycin (1 μ M) and survival was determined as in Figure 1a. Shown are mean \pm s.e.m. for N=9 (untreated and RAPA) or N=3 (AZD) samples (upper panel). Similarly treated CLL samples were subjected to western blot and probed for MCL-1. To exclude caspase-mediated MCL-1 breakdown, 5 μ M Q-VD-OPh was added to all samples during culture when analyzed for MCL-1 levels by western blotting. β -actin was used as a loading control. Western blot representative for N=6. (lower panel). **f.** CLL samples were co-cultured as in Figure 1 in the presence or absence of 1 μ M PI3K inhibitor CAL101 (idelalisib) and survival was determined as in Figure 1a. Shown are mean \pm s.e.m. for N=7 (upper panel). Similarly treated CLL samples were subjected to western blot and probed for MCL-1, as in Fig2e. Western blot representative for N=4.

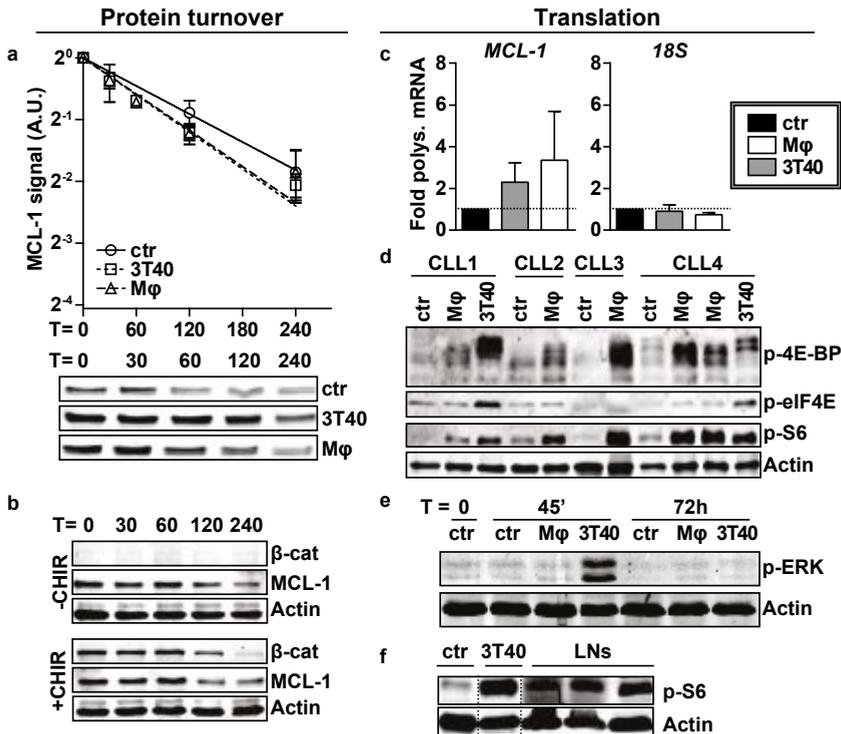


Figure 3: MCL-1 is regulated on translational level. **a.** After co-culture for 72h, turnover of MCL-1 in CLL cells was determined by quantifying levels at different time points after addition of 25µg/mL translation inhibitor cycloheximide, and compared to unstimulated samples (N=6). Each point represents mean \pm s.e.m. and regression lines were calculated using a best-fit exponential decay model fitted with the formula $Y = Y_0 * e^{k*x}$, in which Y_0 was set to 1. The best fit k value was not significantly different between lines (upper panel). A representative turnover western blot for 1 CLL sample is shown (lower panel). Note the higher MCL-1 starting levels in the stimulated samples. A.U. denotes arbitrary units. **b.** Unstimulated CLL samples were pre-treated or not treated for 1h with 3µM GSK3 inhibitor CHIR99021 before MCL-1 turnover was determined as in Figure 3a. To exclude caspase-mediated MCL-1 breakdown, 5µM Q-VD-OPh was added to all samples during culture when analyzed for MCL-1 levels by western blotting. A representative CLL sample of N=3 is shown. **c.** The translational efficiency of MCL-1 mRNA was determined after 48h co-culture by calculating the percentage of MCL-1 mRNA bound in polysomes, by performing qPCR on sucrose gradient-separated non-polysomal and polysomal fractions (see methods and Figure S4a). An MCL-1 and 18S qPCR were performed on pooled samples (polysomal/non-polysomal) and the relative amount of polysomal bound mRNA \pm s.e.m. (N=4 independent experiments) compared to the control condition (fold induction) was calculated for MCL-1 or 18S RNA. **d.** CLL samples used in Figure 1b were again subjected to western blot and probed for phosphorylation of translation initiation factors and phosphorylation of ribosomal protein S6, the latter is indicative of active translation. β -actin was used as a loading control. Note that while the anti-p-4E-BP antibody was expected to detect only the slowest migrating form of 4E-BP, the non- and partially phosphorylated forms are also detected, most likely due to cross-reactivity to the unphosphorylated site. **e.** The western blot presented in Figure 2c was probed for ERK phosphorylation (shown blot is representative of N=4). **f.** Protein lysates from 3 CLL LN samples were analyzed by western blot for the phosphorylation of ribosomal protein S6 as a measure for active translation. An unmatched unstimulated and 3T40-stimulated CLL sample were included for comparison.

in MCL-1 half-life while the upregulation of β -catenin, another GSK3 β substrate, indicated effective GSK3 β inhibition (Figure 3b).

As AKT is furthermore involved in the formation of the translation initiation complex, we evaluated changes in translation of *MCL-1* mRNA, by measuring the amount of mRNA bound in actively translating ribosome chains, or polysomes. Sucrose gradient centrifugation of cell lysates was used²⁵ to separate polysomal and non-polysomal mRNA and determine the fraction of polysomal *MCL-1* mRNA. These analyses showed an increase in polysomal bound *MCL-1* mRNA of approximately 2-fold in 3T40 and 3.5-fold in M ϕ -stimulated samples, indicating an increase in *MCL-1* mRNA translation. The distribution of 18S rRNA, in contrast, was not altered upon exposure to 3T40 cells or M ϕ s, implying that the number of actively translating ribosomes did not change (Figure 3c and S4a for a representative BioAnalyzer profile).

The formation of polysomes is initiated after the phosphorylation of several translation initiation factors that are part of the Eukaryotic translation initiation factor 4 (eIF4) complex²⁶. Association of this complex with mRNA depends on the cap-binding factor eIF4E, which is inhibited by Eukaryotic translation initiation factor 4E-binding protein (4E-BP). The mTOR dependent phosphorylation of 4E-BP releases eIF4E to increase translation initiation²⁷, while ERK dependent phosphorylation of eIF4E enhances formation of the eIF4 complex²⁸. Furthermore, the phosphorylation of ribosomal protein S6, that is part of the 40S ribosome, has been implicated in translation initiation²⁹. Thus, the phosphorylation of eIF4E, 4E-BP, and S6 correlate with an increase in translation²⁷.

We therefore analyzed the phosphorylation status of these proteins and found that stimulation with either M ϕ s or 3T40 cells consistently resulted in upregulation of phosphorylation of 4E-BP and S6. Moreover, 3T40 stimulation also increased eIF4E phosphorylation (Figure 3d). In accordance, ERK was phosphorylated exclusively by 3T40 and not by M ϕ s (Figure 3e). As PIM1 and PIM2 kinases have also been described to correlate with 4E-BP phosphorylation³⁰, we analyzed their expression levels, but found no difference in either *PIM1* or *PIM2* expression upon stimulation (Figure S4b).

Lastly, we used protein lysates from FACS-sorted CLL cells isolated from LNs to investigate whether translation was activated *in vivo* and found phosphorylated S6 in all investigated LNs (Figure 3f). Due to a high background signal in the CLL LN lysates, we were unable to detect p-4E-BP (not shown). The upregulation of MCL-1 in CLL on the same LN samples was previously shown by our group⁷.

In line with our findings of translational stabilization, we found no transcriptional induction of *MCL-1* when analyzing the microarray data sets generated for Figure 2 by any stimulation, in contrast to *BCL-X_L* and *BFL-1*, known targets of NF- κ B (Figure S4c). In agreement, after M ϕ co-culture, no NF- κ B subunit translocation occurred (Figure S4d), and no NF- κ B DNA binding activity could be detected in CLL cells using ELISA (Figure S4e).

In summary, 3T40 cells nor M ϕ s led to transcriptional upregulation or post-translational stabilization of MCL-1, but both stimuli induced translation of *MCL-1* mRNA by activation of the initiation complex. This translational activation signature was also present in *ex vivo* LN samples.

MCL-1 upregulation and CLL cell survival are CCR1-dependent

We then aimed to identify the M ϕ -produced factor responsible for the observed survival increase and MCL-1 upregulation via AKT. Various M ϕ -secreted factors have been described to induce AKT signaling, among which are growth factors^{31,32}, integrin signals³³, and chemokines³⁴. As trans-well culturing experiments showed M ϕ -mediated survival in the non-contact setting (Figure S5), we focused on soluble factors for their ability to induce CLL cell survival, while not excluding contact-dependent factors. Using several recombinant growth factors, we could not recapitulate the M ϕ survival effect (Figure 4a). To test the effect of integrin signaling, CLL cells were cultured on plates pre-coated with fibronectin or VCAM, that stimulate several integrin receptors³⁵. As integrin signaling has been described to affect growth factor signaling³⁶, these stimulations were performed with or without a combination of the growth factors used in Figure 4a. Again, none of these stimuli induced survival in CLL cells (Figure 4b). Furthermore, inhibition of integrin signaling in M ϕ -stimulated samples did not affect survival (data not shown). Lastly, highly specific chemokine inhibitors for CCR1, CCR2, and CCR5 were tested in the context of M ϕ -stimulated CLL cells. Of these, inhibition of only CCR1 lead to a complete abrogation of the M ϕ -induced survival effect (Figure 4c). This survival reduction was not the result of non-specific cytotoxic effects, as unstimulated cell survival was not affected by CCR1 inhibition. We verified the effect of CCR1 inhibition in 9 CLL samples, and moreover included 3T40-stimulated CLL cells. Again, CCR1 inhibition completely negated the M ϕ -mediated CLL cell survival, whereas it had no effect on 3T40-mediated survival (Figure 4d). The absence of an effect in the context of 3T40 stimulation likely results from the concurrent upregulation of BCL-X_L and BFL-1 after 3T40 stimulation (Figure 1b).

We then investigated whether the reduction in survival was mediated via MCL-1 and found that in both M ϕ and 3T40-stimulated CLL cells, CCR1 inhibition led to a strong reduction in MCL-1 protein levels. In addition, the phosphorylation of 4E-BP was reduced after CCR1 inhibition, indicating involvement of AKT-mTOR signaling in CCR1-mediated MCL-1 induction.(Figure 4e)

As several chemokine ligands have been described to signal via CCR1³⁷, we tested if a combination of rhCCL3, CCL5 and CCL23 could mimic the M ϕ survival effect. As chemokine signaling can be dependent on presentation via heparin sulphate proteoglycans (HSPGs), these experiments were also performed using feeder layers of VCAM or NIH-3T3 cells, that express HSPGs. However, no survival benefit of the recombinant proteins was observed (Figure 4f), suggesting that the relevant chemokine acts in conjunction with a second M ϕ -secreted factor.

Altogether, these data indicate a dependence on CCR1 in both M ϕ - and CD40L-mediated survival.

DISCUSSION

In this article, we investigated how two important micro-environmental signals, CD40L and macrophages (M ϕ s), lead to upregulation of key anti-apoptotic protein MCL-1 in primary leukemic cells, studying both extracellular and intracellular factors. In contrast to our starting assumption that MCL-1 in CLL cells is mostly post-translationally regulated,

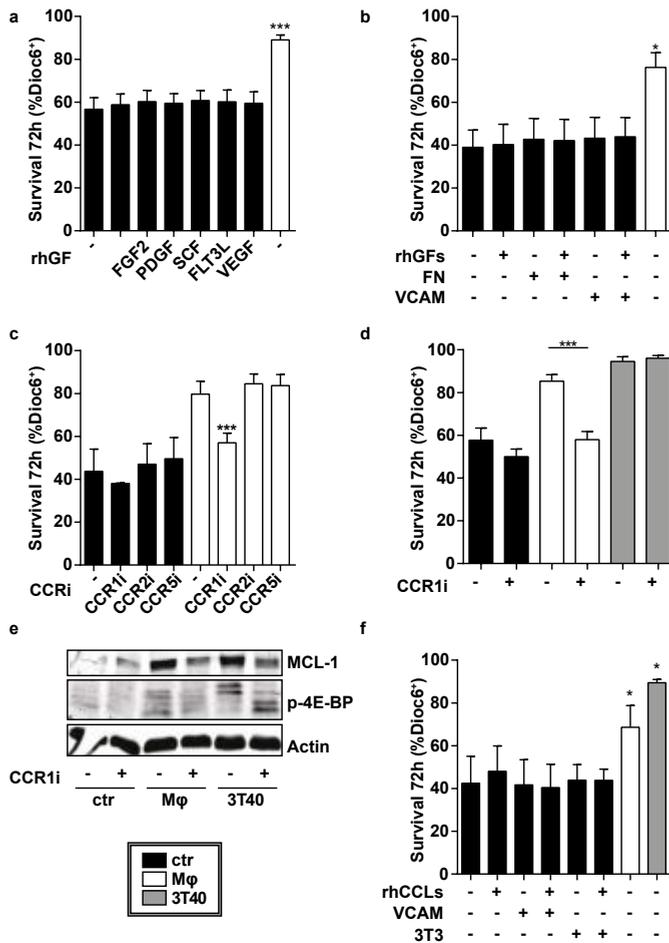


Figure 4: MCL-1 upregulation and CLL cell survival are CCR1 dependent. **a.** CLL samples were cultured in the presence of 25ng/mL Epidermal growth factor (EGF), 20ng/mL Basic fibroblast growth factor (FGF2), 10ng/mL Platelet-derived growth factor (PDGF), 50ng/mL Stem cell factor (SCF), 25ng/mL Fms-related tyrosine kinase 3 ligand (FLT3L), 10ng/mL Vascular endothelial growth factor (VEGF), or nothing or on Mφ for 72h and survival was measured. Shown are mean ± s.e.m. for N=6 CLL samples. **b.** CLL cells were cultured without feeder layer, or on fibronectin coated plates (FN), on VCAM coated plates (VCAM), or on Mφ for 72h in the presence of absence of a combination of the growth factors (rhGFs) used in Figure 5a. Survival is shown as mean ± s.e.m. for N=3 CLL samples. **c.** CLL cells were co-cultured on Mφ or without feeder layer (ctr) for 72h in the presence of absence of specific CCR inhibitors against CCR1 (1μg/mL BX471), CCR2 (100ng/mL INCB3284), or CCR5 (1μM Maraviroc). Next, survival was measured as in Figure 1a. Shown are mean ± s.e.m. for N=3 CLL samples. **d.** CLL cells were co-cultured on indicated feeder layers or without feeder layer (ctr) for 72h in the presence of absence of CCR1 inhibitor BX471. Next, survival was measured as in Figure 1a. Shown are mean ± s.e.m. for N=9 CLL samples. **e.** After 72h co-culture as in Figure 3d, protein lysates of CLL cells were probed by western blot for MCL-1 and 4E-BP. Data shown for 1 patient is representative of N=3. **f.** CLL cells were co-cultured on indicated feeder layers for 72h or cultured with a combination of CCR1-binding chemokines CCL3, 5, and 23 on non-coated or VCAM-coated plates or on 3T3 cells. Next, survival was measured as in Figure 1a. Shown are mean ± s.e.m. for N=3 CLL samples comparing each feeder layer with to without chemokines or Mφ or 3T40 to control.

we determined that both M ϕ and CD40L stimulation induced MCL-1 via AKT-dependent activation of the translation initiation complex (Figure 5). Several observations support the notion that activation of the initiation complex results in a specific translational increase of MCL-1. First, mRNAs with long GC-rich highly structured 5' UTRs, such as the mRNA from MCL-1, are particularly sensitive to translational regulation³⁸. Second, in the Tsc2(+/-) E μ -Myc mouse model, which has constitutive Akt activation, translationally induced Mcl-1 appeared to be the main determinant of mTOR dependent survival³⁹. Third, the survival effect that resulted from expression of constitutively active AKT (myr-AKT) in CLL cells, could be reverted by downregulation of MCL-1 by siRNAs⁴⁰. Fourth, overexpression of a phosphomimetic S209D eIF4E variant in cancer cell lines selectively increases the translation of a limited number of proteins, among which is MCL-1⁴¹. Fifth, inhibition of the translation complex following glucose deprivation sensitizes cells to death receptor-mediated apoptosis as a result of translational MCL-1 downregulation⁴². Modification(s) of translation factors could thus be a micro-environmental regulatory mechanism inducing specifically pro-survival proteins such as MCL-1.

In addition to translational control, several reports describe post-translational stabilization of MCL-1 that is mediated via GSK3 β ^{22, 24}. Other reports show an increase in MCL-1 transcription in CLL after co-culture with mesenchymal stromal cells⁴³, or after STAT activation with cytokines⁴⁴. Our observation of translational regulation of MCL-1 independent of GSK3 β adds to the wide spectrum of MCL-1 control. These distinct mechanisms of MCL-1 control are not mutually exclusive and their relevance will probably depend on the cellular context, and apparently post-translational stabilization is not the dominant mechanism in primary CLL cells.

Furthermore, MCL-1 upregulation after CD40L or M ϕ stimulation was independent of NF- κ B activation (Figure S4d-e). In agreement, no consensus NF- κ B binding sites can be found in the *MCL-1* promotor (Figure S4f). Although several publications report a correlation between NF- κ B activation and MCL-1 levels in CLL cells in response to other stimuli^{5, 44, 45}, this may very well be due to autocrine interleukin-mediated MCL-1 upregulating signals that respond to NF- κ B activation⁴⁴, or alternatively, MCL-1 and NF- κ B are both regulated by an upstream activator, as is the case for CD40 signals. In summary, it appears that under certain conditions NF- κ B activation can indirectly induce *MCL-1* transcription.

We have found that the responsible M ϕ factor for CLL survival is a chemokine that signals via CCR1, while excluding growth factors, and integrins. In this light, we have moreover excluded the involvement of A proliferation inducing ligand (APRIL)⁶ and CD40L (data not shown). Chemokines that can signal via CCR1 include CCL3, 4, 5, 7, 14, 15, 16, and 23³⁷. Interestingly, the reduction in MCL-1 levels after CCR1 inhibition of 3T40-stimulated CLL samples suggests that this upregulation of MCL-1 depends on (indirect) chemokine-mediated signals. Indeed, we recently observed production of several chemokines such as CCL3, 5 and 7 by CLL cells after 3T40 stimulation⁴⁶ in support of a model in which MCL-1 upregulation after 3T40 stimulation depends on autocrine chemokine stimulation of CLL cells (Figure 5).

In conclusion, our data indicate that two model systems of important micro-environmental stimuli (CD40L and M ϕ s) are able to induce survival in primary CLL cells by

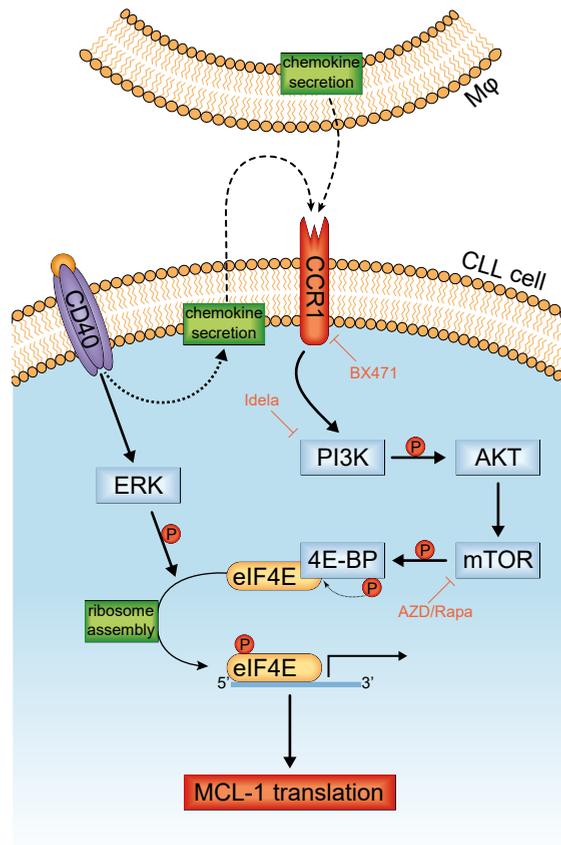


Figure 5: Schematic model for the chemokine-mediated micro-environmental translational regulation of MCL-1. Chemokines secreted directly by macrophages or in an autocrine fashion by CLL cells after CD40 stimulation lead to triggering of the CCR1 receptor. This triggering induces AKT signaling and subsequent induction of mTOR dependent 4E-BP phosphorylation and release of its inhibited binding partner eIF4E. Concurrently, the induction of ERK signaling by CD40 stimulation results in the phosphorylation of eIF4E. These processes facilitate the recruitment of eIF4E to the 5' mRNA cap and formation of the ribosome, which results in the active translation of mRNA of pro-survival proteins like MCL-1.

upregulating MCL-1 translation via AKT signaling. This MCL-1 upregulation resulted from CCR1-mediated signals after both stimuli. These insights may be applicable in designing new treatment strategies for CLL.

MATERIAL AND METHODS

CLL and healthy donor material and isolation

Patient material was obtained from CLL patients, after written informed consent, during routine follow-up or diagnostic procedures at the Academic Medical Center, Amsterdam, the Netherlands. The studies were approved by our Ethical Review Board and conducted

in agreement with the Helsinki Declaration of 1975, revised in 1983. Peripheral Blood mononuclear cells (PBMCs) of CLL patients were isolated using ficoll (Pharmacia Biotech, Roosendaal, The Netherlands) and stored in liquid nitrogen. Expression of CD5 and CD19 (both Beckton Dickinson Biosciences [BD], San Jose, CA) on leukemic cells was assessed by flow cytometry (FACS Canto, BD) and analyzed with FACSDiva software (BD). All samples contained at least 90% CD5⁺/CD19⁺. More information on the characteristics of the CLL patients that provided material can be found in supplemental table 1.

Monocyte derived Mφs were obtained by differentiating monocytes isolated from healthy donor buffy coats after obtaining written informed consent. To this end, PBMCs were isolated using ficoll gradient purification according to manufacturer's instructions (Lucron, Dieren, The Netherlands), after which monocytes were separated from peripheral blood lymphocytes using percoll gradient purification (GE healthcare, Milwaukee, USA). Next, monocytes were incubated to adhere at 37°C in 5% CO₂ for 40min at a concentration of 0.75*10⁶ cells/mL in 6-well plates (3mL) in IMDM/1% Fetal Bovine Serum (FBS, Invitrogen, Carlsbad, CA, USA) and washed to remove non-adherent cells. The monocytes were then differentiated to either M1 using 10ng/mL IFN-γ or M2 using 10ng/mL IL-4 (both R&D systems, Minneapolis, MN, USA) in IMDM supplemented with 10% FBS, 100u/mL Penicillin-100μg/mL Streptomycin (Life Technologies, Austin, TX, USA), 2mM L-glutamine (Life Technologies) and 0.00036% β-mercaptoethanol (Sigma, St. Louis, MO, USA) (IMDM^{+/+}) for 72h.

Cell culture and co-culture experiments

NIH-3T3 mouse embryofibroblasts (3T3 cells) were supplied and characterized (for identity control, cytogenetics, and immunophenotype) by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). To mimic T cell induced CD40 signaling, these 3T3 cells were stably transfected with human CD40L (3T40 cells) as described previously⁸. When used as adherent feeder layer, fibroblasts were irradiated (30Gy) to stop proliferation before being seeded. Mφs were created as described under "CLL and healthy donor material and isolation". After differentiation, Mφs were washed twice with IMDM^{+/+}. CLL samples were thawed and diluted to a concentration of 1.5x10⁶ cells/mL before being plated on the respective feeder cells and co-cultured for indicated times. Trans-well experiments were performed according to manufacturer's instructions using 0.4μm pore size 24-well culture plates (Corning, Corning, NY, USA).

Microarrays and bioinformatic analyses

CLL samples were stimulated with macrophages or not stimulated as described under "Cell culture and co-culture experiments". Total RNA from these stimulated samples was prepared and microarray experiments were performed as they were for 3T40/non-stimulated samples as described before³. In short, RNA was isolated using TriReagent (Sigma) according to manufacturer's instruction and RNA was further purified using the RNeasy micro kit (Qiagen, Valencia, CA, USA) according to manufacturer's instruction including a DNase (Qiagen) treatment. RNA was then hybridized on a U133plus2 microarray chip (Affymetrix, Santa Clara, CA, USA). The macrophage and 3T40 experiments were analyzed separately using Bioconductor packages in the statistical software package R

(version 3.1.2). Raw data were extracted from the CEL files using the package *affy*. Data was normalized and summarized at the probeset level using robust multiarray averaging (RMA) with default settings (function *rma*, package *affy*). Differential expression between the experimental conditions was assessed with a moderated t-test using the linear model framework including patient as a blocking variable (*limma* package). Resulting P-values were corrected for multiple testing using the Benjamini-Hochberg false discovery rate. Corrected P-values ≤ 0.05 were considered statistically significant. Probes were reannotated using the Bioconductor *hgu133plus2.db* package. Data were visualized using the *ggplot2* package. To identify upstream regulators, these differentially regulated genes were used for an Ingenuity pathway analysis using cut-off values of log fold change >0.8 and P-value <0.01 to select relevant regulated genes. For the CAMERA analysis, gene sets were retrieved from MSigDB v5.1 (Hallmark collection, Entrez Gene ID version): <http://www.broadinstitute.org/gsea/msigdb/index.jsp> and probe sets that mapped to 2 or more Entrez Gene IDs were excluded. Next, enrichment analysis was performed using CAMERA with preset value of 0.01 for the inter-gene correlation. The identification of NF- κ B consensus sites was performed using a Python 3 script (supplemental script).

Measurement of levels of polysomal bound MCL-1 mRNA

40×10^6 CLL cells were co-cultured for 48h on M ϕ s or 3T40 cells or left unstimulated as described under "Cell culture and co-culture experiments". Cells were then lysed in 1mL NP-40 buffer (0.5% NP40, 10mM TrisHCl pH 8.0, 140mM NaCl, 1.5mM MgCl₂) with freshly added DTT (Sigma D0632 20mM), cycloheximide (Sigma C7698 150ng/mL), RNAsin (Promega N2515, 12 μ l/mL), and protease inhibitors (Calbiochem 539131 1:100). Lysates were loaded on linear 40%-15% sucrose gradients and centrifuged for 120min at 38000RPM. Gradients were subsequently separated in 18 fractions and each fraction was incubated for 30min at 37°C with 1% SDS, 10mM EDTA and 150 μ g/mL proteinase K (Roche), after which RNA was isolated from each fraction using phenol-chloroform extraction. To identify the presence of polysomes, 1 μ l of each fraction was run on a 2100 Bioanalyzer (Agilent technologies, Santa Clara, CA, USA). Non-polysomal and polysomal fractions were pooled and equal amounts of RNA were used for cDNA synthesis by reverse transcriptase reaction according to manufacturer's instructions (Promega, Madison, USA). The cDNA was subsequently used as input for a real-time PCR using SYBR green (Life Technologies) reaction (40 cycles of 3sec at 95°C followed by 30sec at 60°C), using the following primers: MCL-1 5'-TCGTAAGGACAAAACGGGAC-3' and 5'-CATTCTGATGCCACCTTCT-3', 18S 5'-CGGCTACCACATCCAAGGAA-3' and 5'-GCTGGAATTACCGCGGCT-3'. The abundance of each RNA was then calculated using the formula

$$\% \text{ polysomal RNA} = \frac{(2^{\Delta} - (\text{CtHF} - \text{CtLF})) / (\text{input HF} / \text{input LF})}{(2^{\Delta} - (\text{CtHF} - \text{CtLF})) / (\text{input HF} / \text{input LF}) + 1} * 100\%$$

where Ct = Ct value of HF = heavy (polysomal) fraction and LF = light (non-polysomal) fraction and input = the volume of RNA solution used for cDNA synthesis. The abundance of polysomal RNA was then plotted relative to the unstimulated condition.

Reagents

Recombinant human proteins were obtained from the following manufacturers: Basic fibroblast growth factor, Platelet-derived growth factor, Fms-related tyrosine kinase 3 ligand, and Vascular endothelial growth factor (all R&D systems), Epidermal growth factor (Sigma), Stem cell factor (Peprotech, Rocky Hill, NJ, USA), CCL3 (R&D systems), CCL5 (R&D systems), CCL23 (BioLegend, San Diego, CA, USA);

The following inhibitors were used: mTOR inhibitors AZD8055 (Selleckchem, Houston, TX, USA) and rapamycin (Cell Signaling, Boston, MA, USA), PI3K inhibitor CAL101 (Selleckchem), caspase inhibitor Q-VD-OPh (Apexbio, Houston, TX, USA), GSK3 inhibitor CHIR99021 (Sigma), CCR1 inhibitor BX471 (Sigma), CCR2 inhibitor INCB3284 (Tocris Bioscience, Bristol, UK), CCR5 inhibitor Maraviroc (Apexbio).

Cell viability assays

To assess cell viability, 100 μ l of CLL cell suspension was incubated with 0.01 μ M Dihexyloxacarbocyanine Iodide (Dioc6, Molecular Probes) for 30-40min at 37°C. Prior to analysis, propidium iodide (PI, Sigma) was added (final concentration 2 μ g/mL). Signals were measured on a FACS Calibur (BD) and analyzed using FlowJo software (TreeStar, San Carlos, CA, USA). Viable cells were defined as being Dioc6+/PI-.

Histology and immunofluorescence

Paraffin embedded tissue was obtained from our institute's pathology department. Four-micron sections were de-waxed by immersion in xylene and hydrated by serial immersion in ethanol and PBS. Antigen retrieval was performed by heating sections for 20min in sodium citrate buffer (10mM sodium citrate, 0.05% Tween20, pH 6.0). Sections were washed with PBS (2 x 10min) and blocking buffer (TBS containing 10% BSA and 0.3% Triton X-100) was added for 1 hour. Sections were incubated with primary antibody, anti-CD20 (1:500, eBioscience, San Diego, CA, USA) and anti-MCL-1 (1:150, Abcam, Cambridge, MA, USA), or CD68 (Biolegend Y1-82A) in blocking buffer overnight at 4°C. Subsequently, the slides were washed with PBS (2 x 10min) and incubated with Alexa Fluor 488 labeled goat anti-mouse, Alexa Fluor 594 labeled goat anti-rabbit antibodies (1:400, Invitrogen) for 1 hour, after which the slides were stained for 10min with DAPI (0.1 μ g/mL in PBS). Alternatively, CD68 was developed using the VECTOR Blue Alkaline Phosphatase (AP) Substrate Kit (VectorLabs, Burlingame, CA, USA) and counterstained with Methyl Green. Sections were mounted with Fluoromount-G (eBioscience) and immunofluorescent imaging was performed using a Leica DMRA fluorescence microscope equipped with a cooled camera. Images were acquired using Image Pro Plus and composed in Adobe Photoshop CS5 (Adobe systems, San Jose, CA, USA).

NF- κ B binding Enzyme-Linked Immuno Sorbent Assay (ELISA)

After washing CLL cells in ice-cold PBS, nuclear lysates were prepared using the NucBuster™ protein extraction kit (Millipore, Billerica, USA) according to manufacturer's instructions. Protein concentration was measured with the BCA protein assay kit (Pierce, Thermo Fisher Scientific, Rockford, IL, USA) and 5 μ g of protein was subsequently used as input for the TransAM® NF- κ B Family Transcription Factor Assay Kit, using provided antibodies for

p65 and p52 according to manufacturer's instructions. Signal intensity at 450nm was then determined by spectrophotometer (Biorad, Hercules, CA, USA).

MCL-1 Knockdown

CLL cells were transfected using the AMAXA nucleofection technology (Amaxa, Cologne, Germany) with one of 2 different *MCL-1* siRNAs or control siRNA according to the manufacturer's instructions and as described⁴⁷. In short, CLL cells were left to recover after thawing for at least 3h at 37°C in 5% CO₂. Cells (5.5x10⁶) were resuspended in 100 µl human B-Cell nucleofector kit solution (Lonza, Basel, Switzerland) and nucleofected with Silencer® Select siRNAs (final concentration 3µM) directed against *MCL-1* or a negative control (Ambion, Paisley, UK; catalog numbers s8585 (2 CLL samples) and s8583 (3 CLL samples) for *MCL-1* and AM4635 as negative control) using program X-001. After transfection, cells were directly resuspended in pre-warmed IMDM^{+/+} and plated in 6-well plates to recover for at least 1h before commencing stimulation.

Western blot and MCL-1 turnover analysis

Sample preparation and western blot were performed as described before⁸, using antibodies directed against MCL-1 (Cell Signaling 4572), BCL-X (BD 610211), BCL-2 (Alexis ALX-210-701-C1), BFL-1 (kind gift from Jannie Borst, Immunology Department, Dutch Cancer Institute, Amsterdam), NF-κB p65 (Santa Cruz 372), NF-κB p100/p52 (Cell Signaling 4882), phospho-4E-BP^{T37/46} (Cell Signaling 2855), phospho-eIF4E^{S209} (Cell Signaling 9741), phospho-S6^{S240/244} (Cell Signaling 5364), phospho-AKT^{S473} (Cell Signaling 4060), phospho-ERK (Cell Signaling 4370), phospho-GSK3β^{S9} (Santa Cruz 11757-R), and Actin (Santa Cruz 1616) and Histon H3 (Cell Signaling 9715). MCL-1 turnover time was determined by densitometric quantification of the MCL-1 western blot bands that were normalized to Actin levels at different time points. After calculating relative expression compared to T=0, exponential decay regression models were fitted using Prism 5.0 software (Graphpad, La Jolla, CA, USA). Presented images were composed in Adobe Photoshop CS5.

Statistical analysis

When comparing multiple groups, an analysis of variance (ANOVA) test with Dunnett's (comparing to control) or Newman-Keuls (comparing all groups) post-hoc tests were performed to test for significant differences between multiple groups. When comparing 2 groups, a Student's t-test was used. P values <.05 (*), <.01 (**), and <.001 (***) were considered statistically significant.

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SUPPLEMENTARY MATERIALS

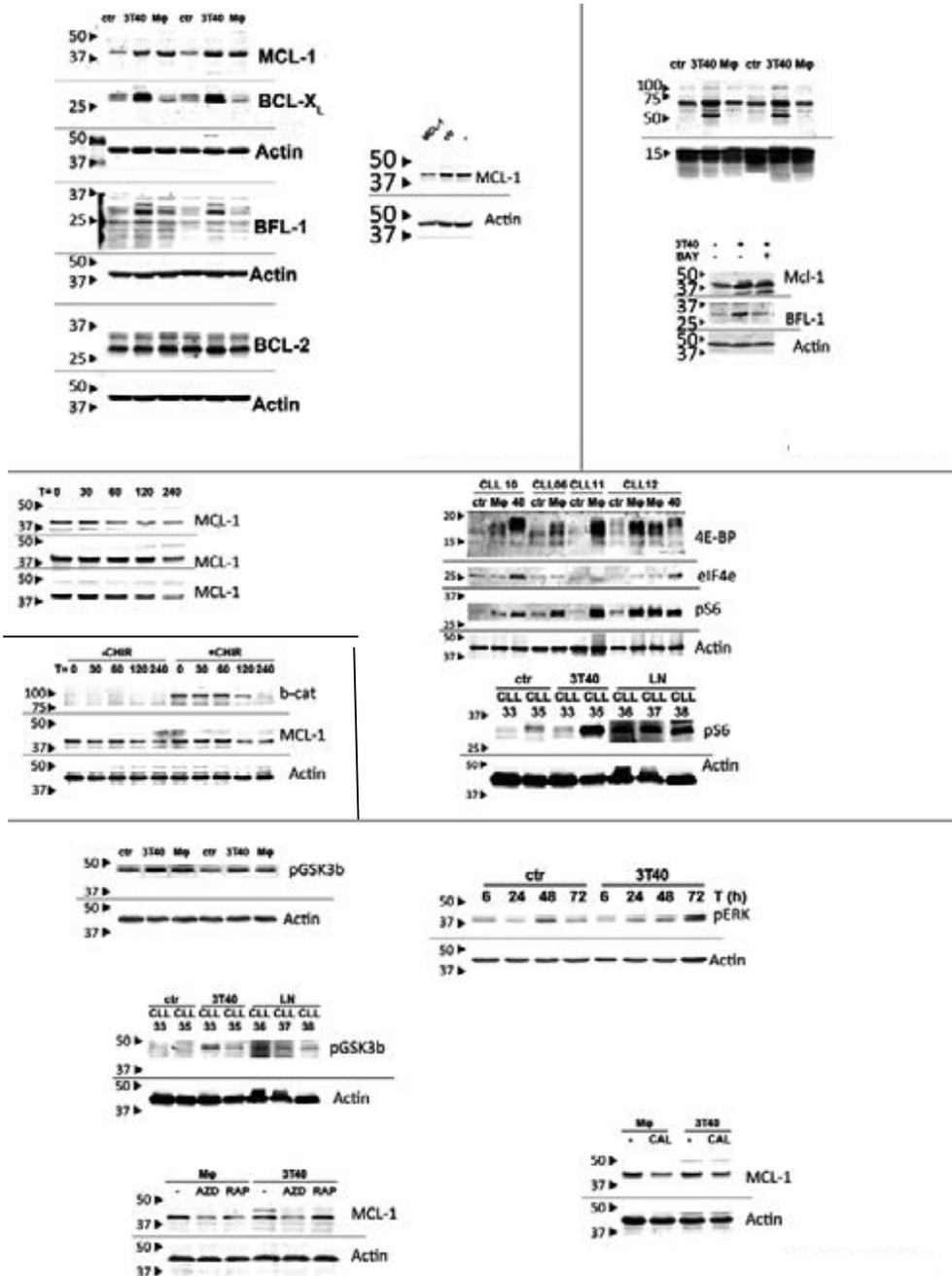


Figure S1: Size marker indication for presented western blots. Molecular size markers are indicated for all western blots presented in the main figures.

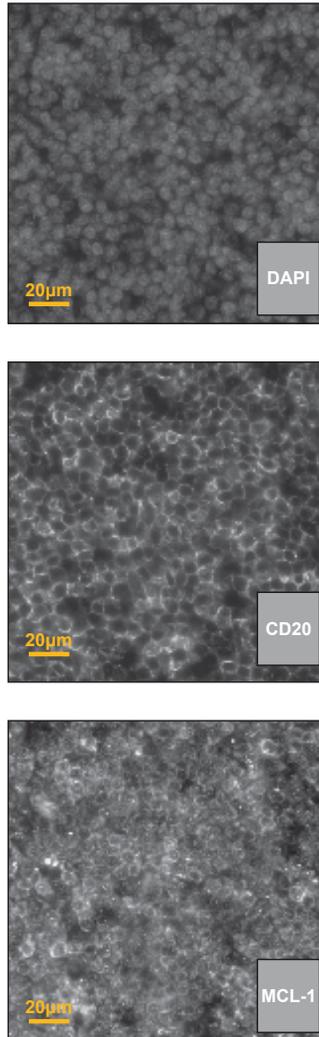


Figure S2: Single channel images of figure 1d, right. Single channel images from the merged color image in figure 1d are presented for DAPI, CD20 and MCL-1.

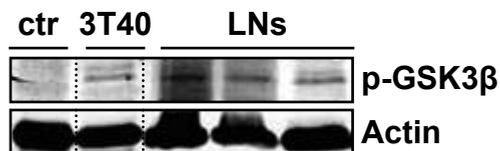


Figure S3: AKT signaling is active in CLL LNs. Protein lysates from 3 CLL LN samples were analyzed by western blot for the phosphorylation of GSK3β. An unmatched unstimulated and 3T40-stimulated CLL sample were included for comparison. β-actin was used as a loading control.

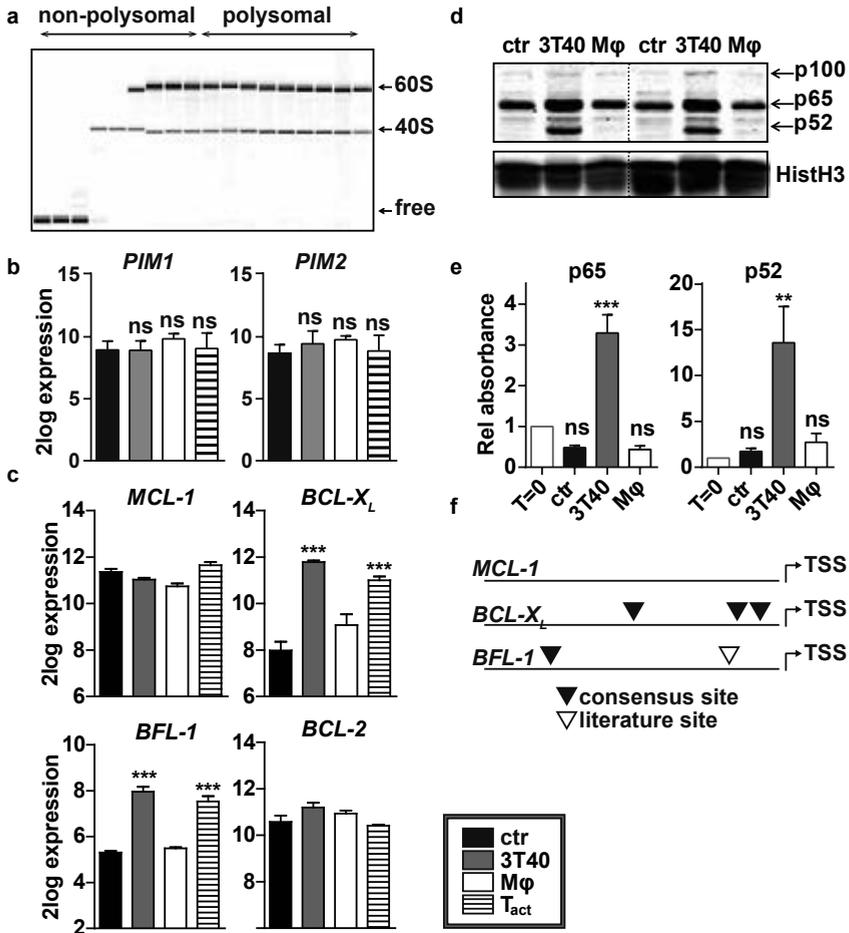


Figure S4: No indications for transcriptional MCL-1 induction or post-translational stabilization. **a.** A representative Bioanalyzer profile for the polysome analysis presented in Figure 3c is shown for 18 sucrose gradient fractions, where left is the top fraction in the gradient. **b.** Expression data generated in Figure 2 was combined with the dataset from Pascutti et al. (3) and analyzed for differential expression of *PIM1* and *PIM2*. Shown are mean \pm s.e.m. for N=11 for ctr, N=13 for 3T40, N=3 for M ϕ , and N=5 for Tact. **c.** Using the same dataset as in Figure S4b, the differential expression of anti-apoptotic BCL-2 family members was analyzed. Shown are mean \pm s.e.m. for N=11 for ctr, N=13 for 3T40, N=3 for M ϕ , and N=5 for Tact. **d.** Nuclei from the same co-cultured CLL samples used in Figure 1b were separated by centrifugation. Nuclear lysates were run on western blot and probed for nuclear translocation of canonical (p65) and non-canonical (p52) NF- κ B subunits. Histone H3 was used as a nuclear loading control. **e.** Similarly as in panel d, nuclear lysates were prepared after 24h co-culture as in Figure 1 to detect the binding of activated NF- κ B subunits p65 and p52 to consensus sequence oligonucleotides by using ELISA. Bars show mean \pm s.e.m. for N=4 in 2 independent experiments for co-cultured samples (as indicated) or samples directly after thawing (T=0) (lower panel). **f.** Promoter sites of *MCL-1* (*Bcl2L3*, NG_029146.1), *BCL-X_L* (*Bcl2L1*, NG_029002.1), and *BFL-1* (*Bcl2A1*, NG_029487.1) were retrieved from the PubMed RefSeq database by obtaining 5000 bases upstream of the transcription start site (TSS) of every gene. Each gene was then analyzed for the presence of the general NF- κ B consensus sequence, (GGGRNYYCC) using a Python 3 script. Each horizontal line represents 5000 bases upstream of the TSS and NF- κ B binding sites are marked to scale.

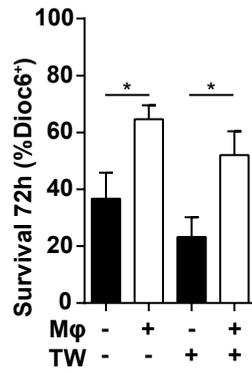


Figure S5: Mφs induce CLL cell survival via a soluble factor. Mφs were seeded as in Figure 1a in the lower chambers of a trans-well culture system and CLL cells were cultured either in direct contact with the feeder cells (TW-), or in the upper chambers of the trans-well inserts (TW+) for 72h after which survival was measured as in Figure 1a. Shown are mean \pm s.e.m. for N=4 CLL samples.

Supplementary script

```

import os          #to scan all files in current directory
cons="GGGRNNYYCC" #consensus sequence

def consscan(cons, promo):
    """scans text (promo) for presence of consensus and returns tuple of positions"""
    lc,lp=len(cons),len(promo)
    return(i-lp for i in range(lp-lc,-1,-1) if patmatch(cons,promo[i:i+lc]))

def patmatch(seq1,seq2):
    """returns boolean whether seq1 matches to seq2. Seq1 also
    accepts wildcards as defined in md (match dictionary)"""
    assert len(seq1)==len(seq2)
    md={"A":"A","C":"C","G":"G","T":"T","R":"AG","Y":"CT","W":"AT","S":"CG",
        "M":"AC","K":"GT","B":"CGT","D":"AGT","H":"ACT","V":"ACG","N":"ATCG"}
    return all(j in md[i] for i,j in zip(seq1,seq2))

def scandir(cons):
    """prints all consensus sites (cons) in all .txt files in the current directory"""
    for file in os.listdir("./"):
        if file.endswith(".txt"):
            with open(file) as fo:
                promo=fo.read()
                assert len(promo)!=0
                filename=fo.name
                sites=", ".join(map(str, consscan(cons,promo)))
                print("Binding positions of {} in {}: {}".format(cons,filename[0:-4],
                    sites if sites else "no match found"))

if __name__ == '__main__':
    scandir(cons)

```

Table S1

ID#	Sample#	Age (years)	WBC count x10 ⁹ /L	CD5/19 (%)	RAI stage	IgVH status	Chromosomal aberrations	Last therapy
1	1046	66	265	99,8	ND	U	13q-	none
3	1226	59	226	93,9	ND	U	None	unkn
4	1175	73	74	94,6	2	M	13q-	>2y
5	1225	79	98	92,4	0	ND	ND	none
6	1178	79	66	88,2	ND	M	ND	none
7	1202	59	180	95,1	ND	U	None	unkn
8	1235	72	134	95,9	1	ND	11q-	>2y
9	1228	64	57	95,2	ND	M	ND	none
10	1047	86	149	98,2	1	M	11q- 13q-	>2y
11	1188	69	ND	ND	ND	U	T12	1y
12	1196	88	30	91,3	0	M	None	>2y
13	1288	65	121	95,5	0	U	13q-	none
14	1327	81	170	95,9	2	U	ND	>2y
15	1285	68	95	92,2	0	M	None	none
16	1122	68	155	79,0	3	U	T12 17p-	1y
17	1367	66	88	90,8	0	M	13q-	none
18	1361	82	41,5	91,1	2	ND	ND	none
19	1350	75	98	25,4	ND	ND	None	>2y
20	1193	63	93	95,1	2	M	ND	>2y
21	250	72	146	88,1	3	M	13q-	none
22	1044	69	153	95,7	0	M	13q-	>2y
23	1250	78	90	91,4	0	U	T12	none
24	1317	73	422	92,8	ND	U	17p-	1y
25	1427	75	129	90,4	ND	U	T12	>2y
26	1480	68	109	90,0	0	M	None	>2y
27	1407	79	216	95,9	ND	ND	ND	none
28	1465	66	124	93,6	0	M	13q-	none
29	1612	64	89	91,8	0	M	13q-	none
30	1619	78	54	92,2	0	ND	None	none
31	1631	67	49	96,7	0	ND	ND	none
32	1633	88	230	97,7	ND	ND	ND	none
33	1429	66	105	86,5	0	M	None	none
34	1464	72	ND	95,9	1	ND	17p-	8mths
35	1481	80	54	92,0	2	ND	ND	none
36	H68-193	47	ND	ND	ND	ND	ND	none
37	782	66	60	99,7	2	M	11q- 13q-	none
38	794	61	75	99,7	ND	U	ND	unkn
39	1551	60	89	97,5	0	M	13q-	unkn

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**GENE EXPRESSION EFFECTS IN CHRONIC
LYMPHOCYTIC LEUKEMIA CELLS
BY MACROPHAGE AND CD40L STIMULATION
INDICATE OXPHOS CONDITIONING**

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ABSTRACT

Chronic lymphocytic leukemia (CLL) cells depend on interactions with bystander cells such as T cells or macrophages for their survival. These interactions with surrounding cells lead to immune suppression by largely unknown mechanism(s). Survival effects are mediated via changes in the apoptotic balance, but changes in other signaling pathways are relatively uncharacterized. Recent reports highlight that immune activation or suppression involves changes in metabolism, but the effects of T cells and macrophages on CLL cell metabolism have not been studied. Using distinct sets of previously generated gene expression profiles of macrophage- and T cell factor CD40L-stimulated CLL cells, we conclude that both macrophages and CD40 stimulation lead to upregulation of oxidative phosphorylation (oxphos) in CLL cells. Both stimuli also induced the PI3K-AKT-mTOR pathway. Considering that increased oxphos could in turn cause immune suppression via ROS production, these data reveal an interesting link between microenvironmental signaling and generalized immune suppression. Further investigation of these pathways could uncover novel therapeutic targets to aid in restoring immune function in CLL.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) cells critically depend on interactions with bystander cells, such as stromal cells, T cells and macrophages¹, in the lymph node. Bystander cells have been shown to induce proliferation, survival, and chemo resistance in tumor cells¹, and these latter two effects are mediated via changes in the apoptotic balance². The tumor-supportive capacity of macrophages is thought to depend on their differentiation status; M1 immunogenic macrophages are considered to be anti-tumorigenic, while M2 tissue repair macrophages are tumor-supportive³. The supportive effects of T cells are largely mediated via CD40L-dependent upregulation of pro-survival B-cell lymphoma 2 (BCL-2) family members proteins, such as BCL2-related protein A1 (BFL-1), B-cell lymphoma-extra large (BCL-X_L) and induced myeloid leukemia cell differentiation protein (MCL-1)⁴. However, we have recently established that both M1 and M2 macrophages predominantly induce survival via upregulation of MCL-1 only⁵.

In order to maintain cell viability while also creating new biomass to proliferate, cancer cells frequently display reprogramming of key metabolic pathways⁶. Therefore, it has been suggested that CLL cells also rely on adaptations of their metabolism next to changes in the apoptotic balance for their survival⁷. Indeed, CLL cells have increased mitochondrial mass and display enhanced oxidative phosphorylation (oxphos) compared to normal B cells⁸. It has been furthermore shown that CLL cells readily change their metabolism in response to extracellular stimuli such as hypoxia⁹ or contact with stromal cells¹⁰. The effects of macrophages and T cells (CD40L) on CLL cell metabolism have however not been studied.

In two previous studies, we have generated gene expression profiles (GEPs) of CLL samples stimulated with M1- or M2-differentiated macrophages⁵ or with CD40L⁴. Using these GEPs, we here studied 1) potentially relevant genes regulated by macrophage stimulation, 2) how the IFN α pathway is activated by CD40L-overexpressing cells, and 3) whether CLL cell metabolism and specifically the oxphos pathway change upon stimulation.

RESULTS

Macrophage stimulation has large transcriptional effects on CLL cells

We first generated a principal component analysis (PCA) plot, combining the GEP data from both experiments (Figure 1A). We found high per-patient clustering, which could obscure stimulation effects in subsequent analyses. When removing the patient effect using a linear model and performing PCA on the residuals, samples clustered per stimulation (Figure 1B). We observed a substantial difference in the PC2 component between the control samples from the macrophage and CD40L experiments (Figure 1B). We therefore refrained from combining data from both experiments and determined differentially expressed genes (DEGs) on a per-experiment basis. These analyses indicated 4296 DEGs for M1-stimulated samples, 1903 for M2, and 3786 for CD40L using a cut-off of <0.01 for the adjusted P value. In line with the upregulation of the AKT and IFN α pathway found previously⁵, the top 100 upregulated genes induced by both M1 and M2 macrophage stimulation included genes involved in AKT signaling (EGR-1, SKP2), IFN α signaling (IFI6, IFIT1), but also other potentially relevant genes (MEX3C, ZBTB24) and several hemoglobin genes

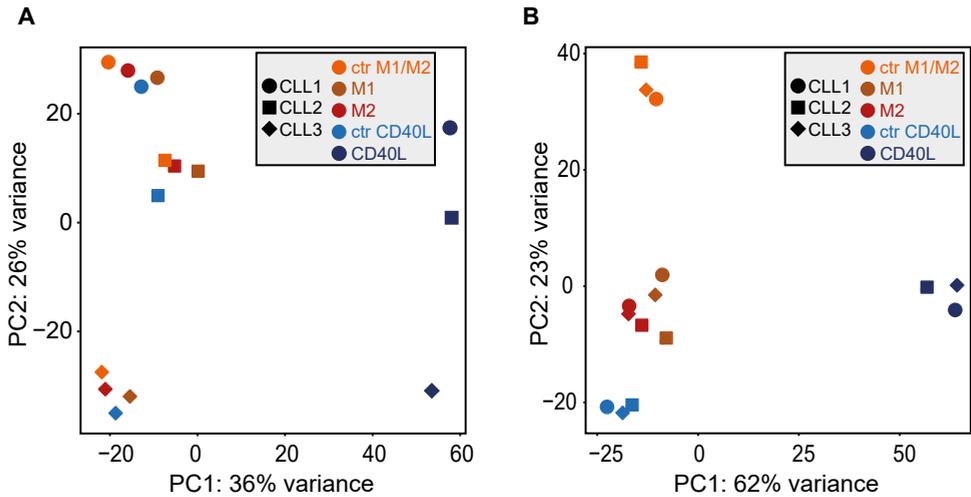


Figure 1: Principal component analysis before and after removal of patient effect. **A.** GEPs from the macrophage experiment were subjected to PCA. Shown are principal component 1 (PC1) and 2 (PC2). The percentage of variance explained by each principal component is indicated on each axis. **B.** PCA plot after removal of the patient effect via a linear model.

(Table S1). MEX3C is an RNA-binding E3 ubiquitin ligase associated with RIG-I-mediated antiviral immunity¹¹. As hemoglobin is not expressed in CLL cells, we assume that the apparent downregulation of several hemoglobin genes in macrophage compared to control samples is an artefact caused by erythrocyte aggregation in these control samples. Given the high overlap between the top genes from M1- and M2-stimulated samples, subsequent analyses were performed on the GEPs from samples stimulated with M1 macrophages (hereafter called macrophages).

Induction of the IFN α pathway after CD40L stimulation is most likely mediated via autocrine IFN α signaling

In our previous study⁵ we found that CD40L induced activation of the IFN α pathway, based on Ingenuity pathway analysis and CAMERA¹² analysis using the MSigDB gene set collection. Indeed, overlaying genes from the IFN α response MSigDB hallmark gene set on a volcano plot indicated that next to macrophage-stimulated CLL cells, CD40L-stimulated cells also overexpress several genes from this gene set (Figure 2A,B respectively). These results are unexpected considering that these CD40L-overexpressing cells do not secrete human IFN α . We tested three hypotheses that could explain this observation. First, the IFN α gene signature might be induced directly by CD40L stimulation. To test this hypothesis, we compared the IFN α gene signature with the CD40 gene signature described by Basso et al. These signatures show very little overlap, suggesting that CD40L is not expected to induce IFN α genes (Figure 2C). Secondly, we considered the possibility that the CD40L-overexpressing mouse NIH-3T3 feeder cells express IFN α that cross-reacts with the human receptor. A Basic Local Alignment Search Tool (BLAST) comparison of the mouse (NP_034632.2) and human (NP_076918.1) amino acid sequence of IFN α

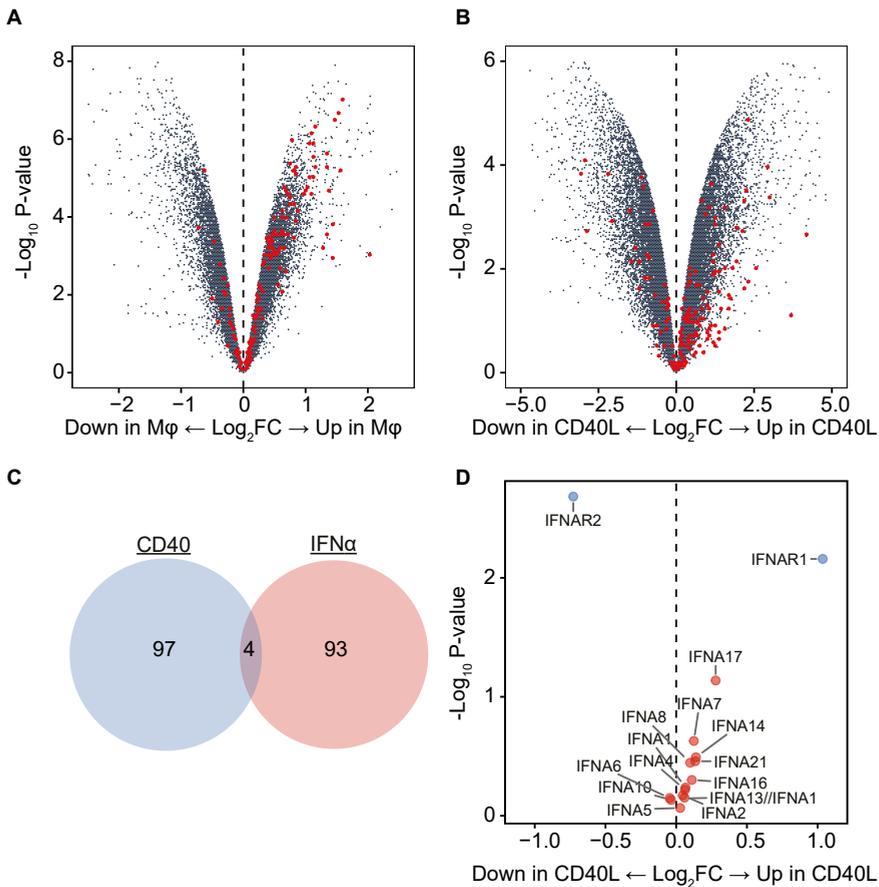


Figure 2: Induction of the IFN α pathway after CD40L stimulation is most likely mediated via autocrine IFN α signaling. **A.** Volcano plot comparing GEPs of macrophage(M ϕ)-stimulated and control CLL samples with genes from the IFN α pathway overlaid in red. **B.** Volcano plot comparing GEPs of CD40L-stimulated and control CLL samples with genes from the IFN α pathway overlaid in red. **C.** Genesets for IFN α - (HALLMARK_INTERFERON_ALPHA_RESPONSE) and CD40- (BASSO_CD40_SIGNALING_UP) stimulated cells were retrieved from MSigDB and the difference and overlap between both sets was visualized in an Euler diagram. Sets and subsets are thus drawn to scale. **D.** Volcano plot of IFN α ligands and receptors for the GEPs of CD40L-stimulated compared to control CLL samples. When multiple probesets for one symbol were present, the probeset with the highest average expression is shown.

however showed 63% overlap (data not shown), indicating that it is unlikely to react with the human IFN α receptor. Thirdly, we investigated if CD40L stimulation leads to upregulation of mRNA for IFN α ligands or receptors in CLL cells, which could lead to autocrine signaling. Although not significant (ROAST gene set test, PMID:20610611; data not shown), we found a trend towards expression of several IFN α ligands and a strong overexpression of chain 1 of the IFN α -R, but downregulation of chain 2 (Figure 2D). Altogether, these results suggest that autocrine IFN α signaling of CLL cells is the most likely cause of IFN α pathway activation after CD40L stimulation.

Both CD40L and macrophage stimulation lead to activation of oxidative phosphorylation in CLL cells

As the effects of CD40L and macrophage stimulation on CLL cell metabolism are currently unknown, we studied the gene expression levels of downstream oxphos gene by analyzing the regulation of the oxphos MSigDB hallmark geneset. A large number of genes of this geneset were upregulated after either macrophage or CD40L stimulation (Figure 3A,B respectively), suggesting an induction of the oxphos pathway.

We then investigated whether functional oxphos genes were also upregulated by visualizing the regulation of oxphos components of the KEGG reference pathway. We found upregulation of all multiunit core enzymes of the electron transport chain by both stimuli (Figure 3C). Moreover, individual molecules from these complexes such as NADH dehydrogenase and ATPase were upregulated by both stimuli, although CD40L led to downregulation of some of these (Figure 3C).

DISCUSSION

We have here addressed the effects of CD40L and macrophage stimulation of CLL cells by studying gene expression signatures generated in two previous studies^{4,5}. On pathway level, we previously found upregulation of the AKT and IFN α signaling⁵. Studying individual differentially regulated genes, we now identified novel genes that are not related to these pathways, but could be relevant contributors to macrophage-mediated effects on CLL cells. MEX3C for instance has been shown to be involved in cytokine production (including of type-1 interferons)¹¹, which might also contribute to autocrine CLL survival signaling. It furthermore induced HLA-1 mRNA degradation¹³ and could thus contribute to inefficient CLL antigen recognition by cytotoxic T cells. ZBTB24, which was also upregulated, is a transcriptional repressor that is involved in cell cycle progression in B cells¹⁴. Bi-allelic mutations in its zinc finger domain are associated with B cell dysfunction and a combined immunodeficiency syndrome¹⁵.

Both CD40L- and macrophage-stimulated CLL cells showed an activation of the IFN α pathway. After CD40L stimulation, IFN α pathway activation likely resulted from autocrine signaling, which is in line with our recent data indicating activation of a cytokine-producing program in CLL cells upon CD40L stimulation⁵. Whether this induction also contributes to CLL cell survival is not known. Although IFN α has anti-tumor properties in a number of malignancies¹⁶, it also upregulates C-C chemokine receptor type 1¹⁷, the receptor on which macrophage-mediated survival depends⁵. Considering that macrophage-mediated survival strongly depended on PI3K-AKT-mTOR signaling⁵, we think IFN α signaling, although present, plays a subordinate or indirect role in survival induction.

Oxidative phosphorylation is the process in which NADH generated from the citric acid cycle is converted to energy in the electron transport chain. In this process, multi-enzyme complexes located in the mitochondrial inner membrane transfer electrons derived from nicotinamide adenine dinucleotide (NADH) while generating a cross-membrane proton gradient that is used for the synthesis of adenosine triphosphate (ATP)¹⁸. We found that both CD40L and macrophage stimulation induced a signature compatible with activation of the oxphos pathway, as each of the core multi-enzyme complexes is upregulated

factors induce oxphos signaling fit with a model in which CLL turnover takes place by virtue of the TME. Although these preliminary findings would still have to be confirmed on the functional level, they are in line with a previous observation where we found an increased mitochondrial mass and oxygen consumption rate in CLL cells after CD40L stimulation (data not shown). Whether the increased mitochondrial mass is the result from an increase in size or quantity of mitochondria, has still to be determined. The observed activation of oxphos would increase the already present upregulation by non-stimulated CLL cells²⁰. This over-activation could lead to immune suppression via excess reactive oxygen species production²⁰. Moreover, oxphos induction can lead to therapy resistance; it has been shown in CLL that oxphos upregulation does not lead to increased ATP production, but rather leads to *de novo* pyrimidine biosynthesis as a result of overproduction of its intermediary metabolites²¹. This could in turn gives rise to resistance towards nucleoside analogs such as fludarabine²¹. Which intracellular pathways induce oxidative phosphorylation in CLL is currently unknown, but the activated PI3K-AKT-mTOR pathway after either CD40L and macrophage stimulation⁵ is a strong candidate. Inhibiting the PI3K-AKT-mTOR pathway might therefore not only reduce MCL-1 levels, but also restore normal metabolism in CLL cells.

METHODS

CLL cell and monocyte isolation and stimulation

Patient material was obtained from CLL patients, after written informed consent, during routine follow-up or diagnostic procedures at the Academic Medical Center, Amsterdam, the Netherlands. The studies were approved by our Ethical Review Board and conducted in agreement with the Helsinki Declaration of 1975, revised in 1983. Peripheral Blood mononuclear cells (PBMCs) of CLL patients were isolated using ficoll (Pharmacia Biotech, Roosendaal, The Netherlands) and stored in liquid nitrogen. Expression of CD5 and CD19 (both Beckton Dickinson Biosciences [BD], San Jose, CA) on leukemic cells was assessed by flow cytometry (FACS Canto, BD) and analyzed with FACSDiva software (BD). All samples contained at least 90% CD5⁺/CD19⁺. Samples from 3 CLL patients were, in two different experiments, stimulated for 16h with either CD40L overexpressing NIH-3T3 cells⁴ or macrophages⁵ or left untreated and CLL cells were sorted to >99% purity using CD5/CD19 staining and FACS.

Microarrays and raw data generation

CLL samples were stimulated as described under "CLL cell and monocyte isolation and stimulation". Total RNA from these samples was prepared and microarray experiments were performed as described before⁴. In short, RNA was isolated using TriReagent (Sigma) according to manufacturer's instruction and RNA was further purified using the RNeasy micro kit (Qiagen, Valencia, CA, USA) according to manufacturer's instruction including a DNase (Qiagen) treatment. RNA was then hybridized on a U133plus2 microarray chip (Affymetrix, Santa Clara, CA, USA). The macrophage and CD40L experiments were analyzed using Bioconductor packages in the statistical software package R (version 3.1.2). Raw data were extracted from the CEL files using the package *affy*.

Data normalization and differential expression

Data were normalized and summarized at the probeset level using robust multiarray averaging (RMA) with default settings (function `rma`, package `affy`). The package `arrayQualityMetrics` was used to assess the quality of the microarray data both before and after normalization. Based on this analysis all arrays were deemed to be of sufficient quality. Principal component analysis (function `plotPCA`, package `DESeq2`) was performed on the top-1000 most variable genes both before and after removal of the patient effect (function `removeBatchEffect`, package `limma`). Subsequently data of both experiments were normalized and analyzed separately. Differential expression between the experimental conditions (M1- and M2-stimulated versus control and CD40L-stimulated versus control, respectively) was assessed with a moderated t-test using the linear model framework including patient as a blocking variable (`limma` package). Resulting P-values were corrected for multiple testing using the Benjamini-Hochberg false discovery rate. Corrected P-values ≤ 0.05 were considered statistically significant. Probes were reannotated using the Bioconductor `hgu133plus2.db` package. Data were visualized using the `ggplot2` package.

6

Gene set enrichment analysis

For gene set enrichment analysis, gene sets were retrieved from MSigDB v5.1 (hallmark collection, Entrez Gene ID version): <http://www.broadinstitute.org/gsea/msigdb/index.jsp> and probe sets that mapped to two or more Entrez Gene IDs were excluded. Next, enrichment analysis was performed using CAMERA¹² (`limma` package) with preset value of 0.01 for the inter-gene correlation and using the same linear model as above.

Visualizing IFN α related genes

The subset of observations from the expression profiles for which the symbols included the term "IFNA" was selected and subdivided into a receptor (containing "IFNAR") and ligand group. If multiple probe sets were present for the same gene, the probe set with the highest average expression level was selected. Data were subsequently visualized in R using `ggplot2` and labeled with the `ggrepel` package.

Comparison of IFN α and CD40 gene sets

Genesets for IFN α - (HALLMARK_INTERFERON_ALPHA_RESPONSE) and CD40- (BASSO_CD40_SIGNALING_UP) stimulated cells were retrieved from MSigDB. The difference and overlap between these gene sets was calculated using the `setdiff` and `intersect` functions in R and visualized using the `venneuler` package.

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SUPPLEMENTARY MATERIALS

Table S1: Top 100 DEGs in M1 and M2 macrophage-stimulated samples selected based on lowest P-value. The logFC values indicate the log₂ fold change of macrophage- compared to non-stimulated CLL samples and the associated P-Values are given. Probe sets not mapping to a gene symbol according to the hgu133pls2.db were excluded. Two forward slashes indicate a probe set mapping to multiple genes.

		M1/ctr				
	SYMBOL	PROBEID	GENENAME	logFC	P value	adj. P value
1	MEX3C	1556874_a_at	mex-3 RNA binding family member C	3,02	9,15E-11	2,98E-6
2	SKP2	210567_s_at	S-phase kinase-associated protein 2, E3 ubiquitin protein ligase	2,01	2,10E-10	2,98E-6
3	LOC100289230	238456_at	uncharacterized LOC100289230	-2,99	2,45E-10	2,98E-6
4	HBB	217232_x_at	hemoglobin, beta	-4,71	4,12E-10	3,51E-6
5	HBA1//HBA2	211745_x_at	hemoglobin, alpha 1// hemoglobin, alpha 2	-4,84	6,84E-10	4,29E-6
6	EGR1	227404_s_at	early growth response 1	2,26	7,30E-10	4,29E-6
7	HBB	209116_x_at	hemoglobin, beta	-4,98	7,84E-10	4,29E-6
8	HBA1//HBA2	209458_x_at	hemoglobin, alpha 1// hemoglobin, alpha 2	-4,70	9,42E-10	4,57E-6
9	HBA1//HBA2	204018_x_at	hemoglobin, alpha 1// hemoglobin, alpha 2	-4,60	1,06E-9	4,57E-6
10	HBB	211696_x_at	hemoglobin, beta	-4,75	1,09E-9	4,57E-6
11	HBA1//HBA2	217414_x_at	hemoglobin, alpha 1// hemoglobin, alpha 2	-4,75	1,25E-9	4,87E-6
12	LPAR6	218589_at	lysophosphatidic acid receptor 6	-2,01	2,83E-9	1,03E-5
13	IFIT1	203153_at	interferon-induced protein with tetratricopeptide repeats 1	2,06	3,05E-9	1,03E-5
14	BTG1	1559975_at	B-cell translocation gene 1, anti-proliferative	2,83	3,22E-9	1,03E-5
15	THRAP3	242163_at	thyroid hormone receptor associated protein 3	-2,14	4,12E-9	1,17E-5
16	HBA1//HBA2	211699_x_at	hemoglobin, alpha 1// hemoglobin, alpha 2	-4,61	4,49E-9	1,17E-5
17	CACNA1A	214933_at	calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	1,64	5,24E-9	1,30E-5
18	HBA1//HBA2	214414_x_at	hemoglobin, alpha 1// hemoglobin, alpha 2	-5,99	6,57E-9	1,51E-5

Table S1: (continued)

	SYMBOL	PROBEID	GENENAME	logFC	P value	adj. P value
19	IFI6	204415_at	interferon, alpha-inducible protein 6	2,22	6,62E-9	1,51E-5
20	ZBTB24	1554036_at	zinc finger and BTB domain containing 24	3,88	8,95E-9	1,81E-5
21	ID2//ID2B	213931_at	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein// inhibitor of DNA binding 2B, dominant negative helix-loop-helix protein (pseudogene)	-2,37	1,03E-8	2,01E-5
22	CABLES1	1558621_at	Cdk5 and Abl enzyme substrate 1	-1,37	1,07E-8	2,01E-5
23	IL7R	226218_at	interleukin 7 receptor	-3,10	1,21E-8	2,20E-5
24	EPC1	238633_at	enhancer of polycomb homolog 1 (Drosophila)	1,47	1,29E-8	2,25E-5
25	BCOR	223915_at	BCL6 corepressor	-1,41	1,31E-8	2,25E-5
26	BCL11B	222895_s_at	B-cell CLL/lymphoma 11B (zinc finger protein)	-2,42	1,55E-8	2,50E-5
27	ZBTB24	1554037_a_at	zinc finger and BTB domain containing 24	3,04	2,64E-8	3,90E-5
28	GPR171	207651_at	G protein-coupled receptor 171	-2,65	2,67E-8	3,90E-5
29	PPP1R3E	227412_at	protein phosphatase 1, regulatory subunit 3E	-1,14	2,85E-8	4,00E-5
30	ZBTB1	1557036_at	zinc finger and BTB domain containing 1	-1,23	3,14E-8	4,13E-5
31	MEX3C	1556873_at	mex-3 RNA binding family member C	2,97	3,20E-8	4,13E-5
32	NSUN7	238983_at	NOP2/Sun domain family, member 7	-1,16	3,46E-8	4,23E-5
33	KLHL6	1560396_at	kelch-like family member 6	1,37	3,48E-8	4,23E-5
34	GIMAP4	219243_at	GTPase, IMAP family member 4	-1,67	3,80E-8	4,52E-5
35	LOC374443	240572_s_at	C-type lectin domain family 2, member D pseudogene	1,65	3,90E-8	4,53E-5
36	ING3	231863_at	inhibitor of growth family, member 3	1,10	4,11E-8	4,69E-5
37	IFRD1	230048_at	interferon-related developmental regulator 1	1,30	4,50E-8	5,02E-5
38	RPS24	1555878_at	ribosomal protein S24	-2,90	4,74E-8	5,18E-5

Table S1: (continued)

	SYMBOL	PROBEID	GENENAME	logFC	P value	adj. P value
39	EIF1	212225_at	eukaryotic translation initiation factor 1	2,79	5,80E-8	5,95E-5
40	SCAF11	1570507_at	SR-related CTD-associated factor 11	-1,25	5,85E-8	5,95E-5
41	ATP5C1	214132_at	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	-1,67	6,00E-8	5,96E-5
42	LOC283788	235535_x_at	FSHD region gene 1 pseudogene	-1,97	6,24E-8	5,96E-5
43	KIAA1429	243927_x_at	KIAA1429	1,12	6,33E-8	5,96E-5
44	SNORA68//RPL18A	1566403_at	small nucleolar RNA, H/ACA box 68//ribosomal protein L18a	1,42	6,52E-8	5,96E-5
45	ITK	211339_s_at	IL2-inducible T-cell kinase	-2,14	6,57E-8	5,96E-5
46	OAS2	228607_at	2'-5'-oligoadenylate synthetase 2, 69/71kDa	1,16	6,77E-8	5,96E-5
47	LOC100272217	243785_at	uncharacterized LOC100272217	-1,54	7,08E-8	6,05E-5
48	BCL10	1557257_at	B-cell CLL/lymphoma 10	2,04	8,07E-8	6,66E-5
49	A2M-AS1	1564139_at	A2M antisense RNA 1 (head to head)	-1,53	8,32E-8	6,66E-5
50	VPS35	1561146_at	vacuolar protein sorting 35 homolog (S. cerevisiae)	-1,31	8,39E-8	6,66E-5
51	TNRC6C-AS1	230526_at	TNRC6C antisense RNA 1	-1,08	8,40E-8	6,66E-5
52	WDFY1	224800_at	WD repeat and FYVE domain containing 1	1,02	8,61E-8	6,73E-5
53	WDFY1	233559_s_at	WD repeat and FYVE domain containing 1	1,29	8,86E-8	6,82E-5
54	CHPT1	230364_at	choline phosphotransferase 1	-1,12	9,10E-8	6,91E-5
55	OAS1	205552_s_at	2'-5'-oligoadenylate synthetase 1, 40/46kDa	1,61	9,45E-8	6,98E-5
56	ABHD13	234993_at	abhydrolase domain containing 13	1,29	1,01E-7	7,40E-5
57	CEP95	243206_at	centrosomal protein 95kDa	1,07	1,13E-7	8,11E-5
58	GTF3A	238880_at	general transcription factor IIIA	-1,18	1,16E-7	8,21E-5
59	PCMTD2	237179_at	protein-L-isoaspartate (D-aspartate) O-methyltransferase domain containing 2	-1,26	1,20E-7	8,38E-5

Table S1: (continued)

	SYMBOL	PROBEID	GENENAME	logFC	P value	adj. P value
60	BCOR	223916_s_at	BCL6 corepressor	-1,12	1,24E-7	8,60E-5
61	RNA45S5	225762_x_at	RNA, 45S pre-ribosomal 5	-2,50	1,27E-7	8,65E-5
62	CD69	209795_at	CD69 molecule	1,71	1,38E-7	8,95E-5
63	FGFBP2	223836_at	fibroblast growth factor binding protein 2	-1,47	1,42E-7	9,03E-5
64	ZNF638	1554248_at	zinc finger protein 638	1,16	1,45E-7	9,03E-5
65	RBMX// SNORD61	1556336_at	RNA binding motif protein, X-linked//small nucleolar RNA, C/D box 61	-1,03	1,47E-7	9,03E-5
66	PAN3-AS1	243092_at	PAN3 antisense RNA 1	-1,57	1,47E-7	9,03E-5
67	SMEK2	1568627_at	SMEK homolog 2, suppressor of mek1 (Dictyostelium)	1,22	1,59E-7	9,39E-5
68	PPP1R3E	229001_at	protein phosphatase 1, regulatory subunit 3E	-1,08	1,59E-7	9,39E-5
69	EGR1	201694_s_at	early growth response 1	2,01	1,60E-7	9,39E-5
70	FLCN	215645_at	folliculin	-1,03	1,68E-7	9,73E-5
71	CIRBP	225191_at	cold inducible RNA binding protein	-2,27	1,74E-7	9,89E-5
72	ZNF540	238454_at	zinc finger protein 540	-1,54	1,84E-7	1,03E-4
73	HPS3	231121_at	Hermansky-Pudlak syndrome 3	1,30	1,94E-7	1,07E-4
74	ID2	201565_s_at	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	-2,20	1,94E-7	1,07E-4
75	PCBP2	213517_at	poly(rC) binding protein 2	-1,06	1,98E-7	1,08E-4
76	UBE2J1	217825_s_at	ubiquitin-conjugating enzyme E2, J1	1,21	2,03E-7	1,10E-4
77	EPSTI1	235276_at	epithelial stromal interaction 1 (breast)	1,52	2,12E-7	1,13E-4
78	PDCD4	1557166_at	programmed cell death 4 (neoplastic transformation inhibitor)	1,32	2,20E-7	1,15E-4
79	GZMH	210321_at	granzyme H (cathepsin G-like 2, protein h-CCPX)	-1,73	2,24E-7	1,15E-4
80	ZNF277	215887_at	zinc finger protein 277	-0,94	2,25E-7	1,15E-4
81	C1orf132	228528_at	chromosome 1 open reading frame 132	-1,18	2,29E-7	1,15E-4
82	LINC01355	228387_at	long intergenic non-protein coding RNA 1355	-1,23	2,44E-7	1,19E-4
83	XAF1	206133_at	XIAP associated factor 1	1,36	2,45E-7	1,19E-4
84	ITPKB	235213_at	inositol-trisphosphate 3-kinase B	-0,95	2,47E-7	1,19E-4
85	ANKRD13D	238642_at	ankyrin repeat domain 13 family, member D	-0,87	2,75E-7	1,32E-4

Table S1: (continued)

	SYMBOL	PROBEID	GENENAME	logFC	P value	adj. P value
86	EPM2AIP1	236314_at	EPM2A (laforin) interacting protein 1	-0,95	2,81E-7	1,33E-4
87	THEMIS2	210785_s_at	thymocyte selection associated family member 2	-0,92	2,81E-7	1,33E-4
88	ATP6AP1L	228816_at	ATPase, H ⁺ transporting, lysosomal accessory protein 1-like	-0,90	2,98E-7	1,39E-4
89	GNG7	228831_s_at	guanine nucleotide binding protein (G protein), gamma 7	-1,27	3,15E-7	1,45E-4
90	CFLAR	210563_x_at	CASP8 and FADD-like apoptosis regulator	1,10	3,24E-7	1,46E-4
91	EPSTI1	227609_at	epithelial stromal interaction 1 (breast)	1,48	3,36E-7	1,49E-4
92	FAM129A	217967_s_at	family with sequence similarity 129, member A	-2,41	3,59E-7	1,57E-4
93	EGR1	201693_s_at	early growth response 1	1,21	3,67E-7	1,59E-4
94	FAM46C	220306_at	family with sequence similarity 46, member C	1,98	3,83E-7	1,65E-4
95	H3F3B// H3F3A// MIR4738	211998_at	H3 histone, family 3B (H3.3B)//H3 histone, family 3A//microRNA 4738	1,56	3,99E-7	1,67E-4
96	HSDL2	1559128_at	hydroxysteroid dehydrogenase like 2	-1,32	4,05E-7	1,68E-4
97	LY9	210370_s_at	lymphocyte antigen 9	-0,92	4,21E-7	1,69E-4
98	THBS1	201110_s_at	thrombospondin 1	-5,30	4,23E-7	1,69E-4
99	NOL12	222057_at	nucleolar protein 12	-1,26	4,23E-7	1,69E-4
100	IREB2	1555476_at	iron-responsive element binding protein 2	1,20	4,27E-7	1,69E-4

Table S1: (continued)

		M2/ctr				
	SYMBOL	PROBEID	GENENAME	logFC	P value	adj. P value
1	MEX3C	1556874_a_at	mex-3 RNA binding family member C	3,45	2,57E-11	7,04E-7
2	LOC100289230	238456_at	uncharacterized LOC100289230	-2,87	3,59E-10	3,23E-6
3	SKP2	210567_s_at	S-phase kinase-associated protein 2, E3 ubiquitin protein ligase	1,88	3,99E-10	3,23E-6
4	BTG1	1559975_at	B-cell translocation gene 1, anti-proliferative	3,51	4,20E-10	3,23E-6
5	HOMER2	1556097_at	homer homolog 2 (Drosophila)	2,32	4,73E-10	3,23E-6
6	HBB	217232_x_at	hemoglobin, beta	-4,38	8,07E-10	4,90E-6
7	HBA1//HBA2	211745_x_at	hemoglobin, alpha 1//hemoglobin, alpha 2	-4,57	1,18E-9	6,33E-6
8	HBB	209116_x_at	hemoglobin, beta	-4,74	1,27E-9	6,33E-6
9	HBA1//HBA2	204018_x_at	hemoglobin, alpha 1//hemoglobin, alpha 2	-4,46	1,42E-9	6,42E-6
10	HBA1//HBA2	209458_x_at	hemoglobin, alpha 1//hemoglobin, alpha 2	-4,47	1,53E-9	6,42E-6
11	HBA1//HBA2	217414_x_at	hemoglobin, alpha 1//hemoglobin, alpha 2	-4,50	2,05E-9	8,00E-6
12	HBB	211696_x_at	hemoglobin, beta	-4,38	2,33E-9	8,38E-6
13	THRAP3	242163_at	thyroid hormone receptor associated protein 3	-2,23	2,91E-9	9,36E-6
14	LPAR6	218589_at	lysophosphatidic acid receptor 6	-1,98	3,28E-9	9,97E-6
15	EPC1	238633_at	enhancer of polycomb homolog 1 (Drosophila)	1,65	4,43E-9	1,21E-5
16	HBA1//HBA2	211699_x_at	hemoglobin, alpha 1//hemoglobin, alpha 2	-4,50	5,55E-9	1,44E-5
17	CABLES1	1558621_at	Cdk5 and Abl enzyme substrate 1	-1,43	7,39E-9	1,76E-5
18	RPS24	1555878_at	ribosomal protein S24	-3,49	8,27E-9	1,88E-5
19	ZBTB24	1554036_at	zinc finger and BTB domain containing 24	3,89	8,78E-9	1,92E-5
20	ID2//ID2B	213931_at	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein//inhibitor of DNA binding 2B, dominant negative helix-loop-helix protein (pseudogene)	-2,37	1,01E-8	2,12E-5

Table S1: (continued)

	SYMBOL	PROBEID	GENENAME	logFC	P value	adj. P value
21	GIMAP4	219243_at	GTPase, IMAP family member 4	-1,89	1,22E-8	2,48E-5
22	ZNF638	1554248_at	zinc finger protein 638	1,51	1,29E-8	2,52E-5
23	LOC283788	235535_x_at	FSHD region gene 1 pseudogene	-2,30	1,49E-8	2,58E-5
24	MEX3C	1556873_at	mex-3 RNA binding family member C	3,22	1,51E-8	2,58E-5
25	PALM2-AKAP2//AKAP2	202760_s_at	PALM2-AKAP2 readthrough//A kinase (PRKA) anchor protein 2	1,79	1,75E-8	2,65E-5
26	HBA1//HBA2	214414_x_at	hemoglobin, alpha 1// hemoglobin, alpha 2	-5,38	1,81E-8	2,65E-5
27	IL7R	226218_at	interleukin 7 receptor	-2,95	1,94E-8	2,65E-5
28	TLE4	233575_s_at	transducin-like enhancer of split 4	1,40	2,10E-8	2,80E-5
29	ING3	231863_at	inhibitor of growth family, member 3	1,17	2,30E-8	2,92E-5
30	LOC374443	240572_s_at	C-type lectin domain family 2, member D pseudogene	1,75	2,30E-8	2,92E-5
31	EIF1	212225_at	eukaryotic translation initiation factor 1	3,07	2,35E-8	2,92E-5
32	ZBTB1	1557036_at	zinc finger and BTB domain containing 1	-1,24	2,79E-8	3,39E-5
33	TLE4	216997_x_at	transducin-like enhancer of split 4	1,48	2,89E-8	3,43E-5
34	CEP95	243206_at	centrosomal protein 95kDa	1,23	3,08E-8	3,46E-5
35	SMEK2	1568627_at	SMEK homolog 2, suppressor of mek1 (Dictyostelium)	1,46	3,10E-8	3,46E-5
36	BCOR	223915_at	BCL6 corepressor	-1,28	3,44E-8	3,69E-5
37	KLHL6	1560396_at	kelch-like family member 6	1,35	3,78E-8	3,90E-5
38	BCL11B	222895_s_at	B-cell CLL/lymphoma 11B (zinc finger protein)	-2,19	3,88E-8	3,93E-5
39	IFRD1	230048_at	interferon-related developmental regulator 1	1,30	4,37E-8	4,34E-5
40	CD69	209795_at	CD69 molecule	1,93	4,48E-8	4,38E-5
41	ATP5C1	214132_at	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	-1,72	4,63E-8	4,42E-5
42	ZBTB24	1554037_a_at	zinc finger and BTB domain containing 24	2,85	4,69E-8	4,42E-5

Table S1: (continued)

	SYMBOL	PROBEID	GENENAME	logFC	P value	adj. P value
43	BRWD1	214820_at	bromodomain and WD repeat domain containing 1	-1,14	5,25E-8	4,85E-5
44	GPR171	207651_at	G protein-coupled receptor 171	-2,46	5,41E-8	4,85E-5
45	GTF3A	238880_at	general transcription factor IIIA	-1,28	5,50E-8	4,85E-5
46	BCL10	1557257_at	B-cell CLL/lymphoma 10	2,11	6,04E-8	5,24E-5
47	H3F3B// H3F3A// MIR4738	211998_at	H3 histone, family 3B (H3.3B)//H3 histone, family 3A//microRNA 4738	1,90	6,48E-8	5,48E-5
48	EGR1	227404_s_at	early growth response 1	1,40	6,69E-8	5,49E-5
49	LOC100272217	243785_at	uncharacterized LOC100272217	-1,51	8,43E-8	6,58E-5
50	ITK	211339_s_at	IL2-inducible T-cell kinase	-2,04	1,01E-7	7,76E-5
51	AKAP2// PALM2-AKAP2	202759_s_at	A kinase (PRKA) anchor protein 2//PALM2-AKAP2 readthrough	1,65	1,03E-7	7,76E-5
52	PHF11	242060_x_at	PHD finger protein 11	-1,14	1,04E-7	7,76E-5
53	IREB2	1555476_at	iron-responsive element binding protein 2	1,39	1,06E-7	7,84E-5
54	SNORA68// RPL18A	1566403_at	small nucleolar RNA, H/ACA box 68//ribosomal protein L18a	1,34	1,08E-7	7,84E-5
55	IL17RB	224361_s_at	interleukin 17 receptor B	1,18	1,11E-7	7,98E-5
56	FLCN	215645_at	folliculin	-1,07	1,25E-7	8,50E-5
57	FGFBP2	223836_at	fibroblast growth factor binding protein 2	-1,49	1,25E-7	8,50E-5
58	SSBP1	214060_at	single-stranded DNA binding protein 1, mitochondrial	0,96	1,26E-7	8,50E-5
59	RNA45S5	225762_x_at	RNA, 45S pre-ribosomal 5	-2,48	1,34E-7	8,70E-5
60	RNF103	1568665_at	ring finger protein 103	3,32	1,38E-7	8,84E-5
61	SCAF11	1570507_at	SR-related CTD-associated factor 11	-1,13	1,51E-7	9,49E-5
62	FAM46C	220306_at	family with sequence similarity 46, member C	2,18	1,59E-7	9,87E-5
63	LINC01355	228387_at	long intergenic non-protein coding RNA 1355	-1,27	1,75E-7	1,05E-4
64	WIPF1	231182_at	WAS/WASL interacting protein family, member 1	1,69	1,77E-7	1,05E-4
65	LOC100506282	220572_at	uncharacterized LOC100506282	-1,39	1,79E-7	1,05E-4
66	CIRBP	225191_at	cold inducible RNA binding protein	-2,26	1,81E-7	1,05E-4

Table S1: (continued)

	SYMBOL	PROBEID	GENENAME	logFC	P value	adj. P value
67	C1orf132	228528_at	chromosome 1 open reading frame 132	-1,20	1,96E-7	1,11E-4
68	PCIF1	222045_s_at	PDX1 C-terminal inhibiting factor 1	1,91	1,98E-7	1,11E-4
69	PAN3-AS1	243092_at	PAN3 antisense RNA 1	-1,52	2,01E-7	1,12E-4
70	RBMX// SNORD61	1556336_at	RNA binding motif protein, X-linked//small nucleolar RNA, C/D box 61	-0,98	2,15E-7	1,17E-4
71	RAB30	206530_at	RAB30, member RAS oncogene family	1,18	2,21E-7	1,20E-4
72	RHOB	212099_at	ras homolog family member B	2,35	2,24E-7	1,20E-4
73	ITPKB	235213_at	inositol-trisphosphate 3-kinase B	-0,96	2,29E-7	1,20E-4
74	ABHD13	234993_at	abhydrolase domain containing 13	1,18	2,29E-7	1,20E-4
75	CHDH	1559590_at	choline dehydrogenase	0,90	2,46E-7	1,27E-4
76	KIAA1429	243927_x_at	KIAA1429	0,97	2,50E-7	1,28E-4
77	A2M-AS1	1564139_at	A2M antisense RNA 1 (head to head)	-1,35	2,59E-7	1,31E-4
78	ZMYM2	210281_s_at	zinc finger, MYM-type 2	0,94	2,80E-7	1,40E-4
79	HSDL2	1559128_at	hydroxysteroid dehydrogenase like 2	-1,38	2,81E-7	1,40E-4
80	CCNL1	1555827_at	cyclin L1	1,69	2,88E-7	1,41E-4
81	ETNK1	224453_s_at	ethanolamine kinase 1	1,22	2,91E-7	1,41E-4
82	MYLIP	228097_at	myosin regulatory light chain interacting protein	1,39	2,92E-7	1,41E-4
83	GPNMB	201141_at	glycoprotein (transmembrane) nmb	1,86	3,16E-7	1,51E-4
84	FOXP1	229844_at	forkhead box P1	0,90	3,29E-7	1,55E-4
85	ETNK1	224454_at	ethanolamine kinase 1	1,90	3,37E-7	1,57E-4
86	TNRC6C-AS1	230526_at	TNRC6C antisense RNA 1	-0,92	3,55E-7	1,60E-4
87	CACNA1D	210108_at	calcium channel, voltage-dependent, L type, alpha 1D subunit	2,48	3,57E-7	1,60E-4
88	PCMTD2	237179_at	protein-L-isoaspartate (D-aspartate) O-methyltransferase domain containing 2	-1,11	3,81E-7	1,67E-4
89	EIF4G3	1554309_at	eukaryotic translation initiation factor 4 gamma, 3	1,22	3,86E-7	1,68E-4
90	TMEM140	218999_at	transmembrane protein 140	-0,88	3,91E-7	1,68E-4
91	DENND4C	234968_at	DENN/MADD domain containing 4C	1,42	3,96E-7	1,69E-4

Table S1: (continued)

	SYMBOL	PROBEID	GENENAME	logFC	P value	adj. P value
92	ZNF154	217242_at	zinc finger protein 154	1,00	3,99E-7	1,69E-4
93	SEC61B	244700_at	Sec61 beta subunit	0,99	4,24E-7	1,77E-4
94	LY9	210370_s_at	lymphocyte antigen 9	-0,91	4,63E-7	1,90E-4
95	BCOR	223916_s_at	BCL6 corepressor	-0,97	4,68E-7	1,91E-4
96	FAM129A	217967_s_at	family with sequence similarity 129, member A	-2,34	4,74E-7	1,92E-4
97	ANKRD13C	1556361_s_ at	ankyrin repeat domain 13C	1,42	4,85E-7	1,94E-4
98	THBS1	201110_s_at	thrombospondin 1	-5,21	4,89E-7	1,94E-4
99	EPM2AIP1	236314_at	EPM2A (laforin) interacting protein 1	-0,89	5,01E-7	1,97E-4
100	VPS35	1561146_at	vacuolar protein sorting 35 homolog (<i>S. cerevisiae</i>)	-1,07	5,19E-7	2,01E-4

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**LENALIDOMIDE UPREGULATES BH3-ONLY
PROTEIN BID IN CHRONIC
LYMPHOCYTIC LEUKEMIA**

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ABSTRACT

Lenalidomide is clinically effective in chronic lymphocytic leukemia (CLL) treatment although it has no direct cytotoxic effects on CLL cells. Its effects are presumed to rather result from immunomodulation. Lenalidomide can induce the degradation of Ikaros proteins, and the importance in the development of healthy B cells of these proteins and their upregulation in CLL cells, led us to investigate whether lenalidomide's clinical efficacy could in addition result from direct effects on CLL cells. Using multiplex PCR, we found that lenalidomide induces upregulation of BH3-only protein BID. These results were verified on protein level and were shown to depend on the ABL1-TP73 axis. Although BID upregulation could theoretically lead to sensitization to death receptor and cell-mediated cytotoxicity, we did not find sensitization to FAS/TRAIL treatment or in cytotoxicity assays. In addition to its effects on BID, lenalidomide upregulated CDK inhibitor p21. This upregulation has been shown to correlate with a decrease in cell proliferation. Lastly, we found an upregulation of Death receptor 6 after lenalidomide treatment. In conclusion, we found several direct effects of lenalidomide on CLL cells, and believe that its proliferation-inhibiting effects most significantly contributes to its clinical efficacy.

INTRODUCTION

Immunomodulatory drugs such as thalidomide and its later developed analogs lenalidomide, pomalidomide, and CC-122 have shown efficacy in the treatment of B cell malignancies such as chronic lymphocytic leukemia (CLL)^{1,2} and multiple myeloma³ during the last decades. Although these drugs have -in addition to their immunomodulatory effects- direct cytotoxic effects towards multiple myeloma cells, no direct cytotoxicity against CLL cells has been shown. Several indirect immunomodulatory effects of lenalidomide can however account for its clinical efficacy in CLL; first, lenalidomide can revert the supportive differentiation of bystander cells, as was shown for monocyte-derived Nurse-like cells⁴. Second, lenalidomide diminishes T cell exhaustion via downregulation of inhibitory receptors such as programmed cell death protein 1 on T cells and restoration of the immune synapse⁵. Third, T cell-mediated CLL killing is potentiated as lenalidomide induces T cell proliferation and cytokine production⁶. T cell killing is furthermore enhanced due to the induction of expression of costimulatory molecules on CLL cells by lenalidomide⁷. Next to these immunomodulatory effects, one study found an effect of lenalidomide on p21-mediated CLL cell proliferation⁸.

Intracellularly, the effects of lenalidomide are mainly mediated via binding to its target cereblon (CRBN)^{9,10}, which is part of an E3-Ubiquitin ligase complex with DNA damage-binding protein 1, Cullin 4A, and regulator of cullins 1⁹. Recently, several groups have independently found that lenalidomide binding leads to altered substrate specificity of CRBN, which results in degradation of transcription factors Ikaros Family Zinc Finger Protein (IKZF)1 and IKZF3 by the proteasome¹¹⁻¹³. The important role of Ikaros transcription factors in non-malignant B cell sustenance^{14,15} and the fact that they are overexpressed in CLL cells¹⁶ suggest that perturbations via lenalidomide treatment could also directly affect CLL cells, albeit in a non-cytotoxic manner. We therefore investigated the effects of lenalidomide on BCL-2 family members and related proteins. Next to confirmation of induction of p21, we found that lenalidomide treatment leads to a transcriptional upregulation of BH3-only protein BID, which could potentially sensitize CLL cells to cell-mediated cytotoxic killing. Furthermore, death receptor DR6 was induced.

RESULTS

Lenalidomide upregulates BID, p21 and DR6

In order to verify the absence of effects of lenalidomide on CLL cell survival, cells were treated for 72h with lenalidomide. CD40L-overexpressing cells were used as a survival-inducing control. As expected, no effect of lenalidomide on CLL cell survival was found (Figure 1A). Considering the fact that Ikaros proteins are upregulated in CLL¹⁶ and that they are able to regulate several BCL-2 family members¹⁷, we next investigated the mRNA levels of 46 apoptosis-related genes using multiplex ligation-dependent probe amplification (MLPA) after lenalidomide treatment. A linear model was fitted on the MLPA data and log fold change and P-value were calculated for each gene and plotted in a volcano plot. We found a strong upregulation of anti-proliferative protein p21^{WAF1/Cip1} (p21) and of death-receptor 6 (DR6). In addition, BH3 interacting-domain death agonist (BID) was upregulated. We did not find an overall shift in the apoptotic balance.

(Figure 1B). Although apparently neither of these upregulated genes induces apoptosis, they have important roles in cell proliferation (p21¹⁸), sensitization to externally induced apoptosis via death receptor signaling (DR6¹⁹ and BID^{20,21}), and granzyme-mediated killing (BID)²². We here focused on the upregulation of BID.

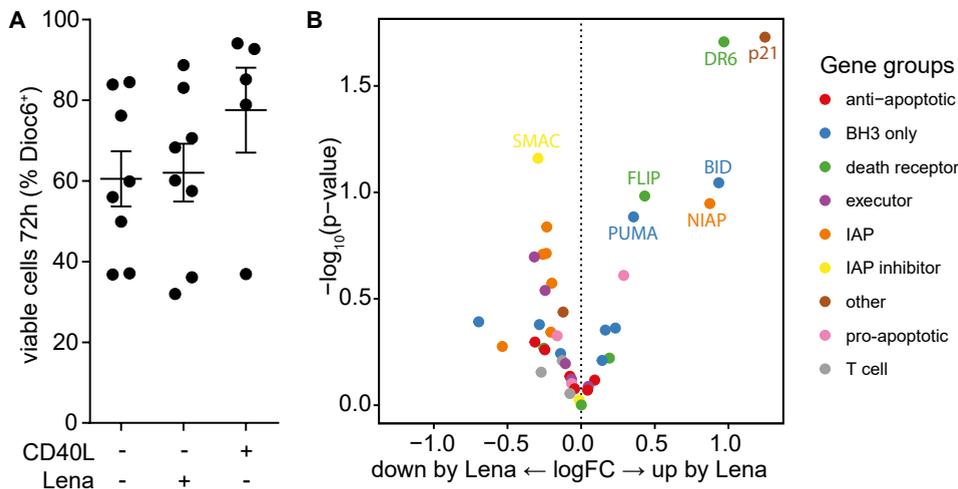


Figure 1: Lenalidomide upregulates BID, p21 and DR6. **A.** CLL cells were treated with 10 μM lenalidomide for 72h, left untreated, or were cultured on CD40L overexpressing NIH-3T3 cells as positive control. Next, the percentage of viable (Dioc6 positive) cells was determined by Dioc6-PI staining. Each dots represents one sample and mean \pm s.e.m. are shown. **B.** CLL cells (N=5) were treated as in Figure 1A for 48h and complete RNA extracts were subjected to RT-MLPA analysis (see methods). mRNA expression levels were then fitted on a linear model using the limma package in R and plotted in a volcano plot. Colors indicate different functional groups contained in the MLPA kit. Highly regulated genes are indicated.

BID is upregulated at therapeutic concentrations

BID upregulation was verified on protein level by western blot together with BCL-2 members Bcl-X_L and MCL-1, which is known to be regulated independent of transcription²³. Whereas MCL-1 and BCL-X_L were unaffected by lenalidomide treatment, BID was strongly upregulated (Figure 2A, B). We then investigated the dynamics of BID upregulation in a time course and found that BID is most strongly upregulated after 72h of treatment (Figure 2C). At this time point, lenalidomide induced BID starting from the clinically attainable²⁴ concentration of 300 nM (Figure 2D). As lenalidomide has been shown to promote CD40L expression on CLL cells²⁵, which could underlie the observed BID upregulation²⁶, we analyzed CD40L levels of lenalidomide-treated CLL cells using flow cytometry, but found no upregulation upon treatment (Figure S1), indicating that its effects are independent of CD40L signaling.

BID upregulation by Lenalidomide depends on TP73 and ABL1 signaling

The transcription factor TP73, which shares many functions with p53, but it is rarely mutated in cancer²⁷, is directly involved in transcriptional regulation of both CDKN1A

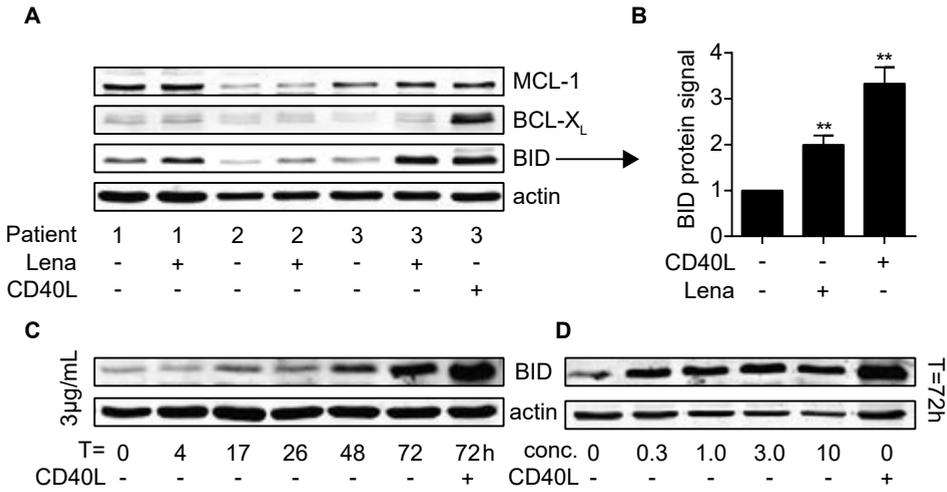


Figure 2: BID is upregulated after extended treatment time at therapeutic concentrations. **A.** CLL cells (N=3) were treated as in Figure 1A and protein extract were subjected to western blot analysis for the indicated BCL-2 family members, and actin was used as a loading control. CD40L stimulated cells were used as a positive BID control. **B.** BID and actin protein levels in CLL samples treated as in Figure 1A were quantified using densitometry and BID/actin ratios were compared to the unstimulated control. Bars show mean \pm s.e.m. for N=5 CLL samples. **, $P < 0.01$ in a t test. **C.** CLL cells were treated with 3 μ M lenalidomide for the indicated times and BID levels were probed by western blot. The western blot shown is representative for N=3. **D.** CLL cells were treated with the indicated concentrations (in μ M) lenalidomide for 72h and BID levels were probed by western blot. The western blot shown is representative for N=3.

(p21)²⁷ and BID²⁶. We therefore studied the involvement of TP73 in lenalidomide induction of BID. Upon treatment of CLL cells with lenalidomide, TP73 was upregulated on protein level concurrently with BID (Figure 3A). Induction of TP73 in solid tumor cell lines depends on post-translational modification mediated by Abelson murine leukemia viral oncogene homolog 1 (ABL1)²⁸. To test the dependence on TP73 in BID regulation, we therefore used the specific ABL inhibitor imatinib. The upregulation of BID and TP73 by lenalidomide could both be reverted when concurrently treating with imatinib (Figure 3A). These results indicate that lenalidomide-mediated BID induction depends on TP73 and that lenalidomide likely acts upstream of both ABL1 and TP73. In line with this, we found that the ABL1 promoter (5000 base pairs upstream of the transcription start site) contained 9 copies of the Ikaros consensus sequence TGGGAW²⁹ (Figure 3B).

BID upregulation by Lenalidomide does not sensitize CLL cells to death receptor or cytotoxic killing

Because BID is a substrate for caspase 8, which is activated upon ligation of death receptors Fas cell surface death receptor (FAS)²⁰ and TNF-related apoptosis-inducing ligand (TRAIL)²¹, we tested if lenalidomide sensitizes CLL cells to FAS or TRAIL-mediated killing using recombinant proteins. FAS ligand (FASL) treatment, also in combination with TRAIL, did not induce CLL cell death, and lenalidomide did not increase the sensitivity of cells to killing (Figure 4A). Next to its potential sensitization in death receptor-mediated

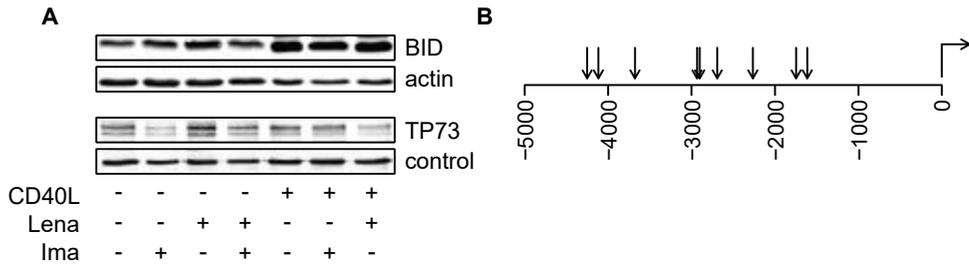


Figure 3: BID upregulation by lenalidomide depends on TP73 signaling. **A.** CLL cells were treated for 72h with the indicated combinations of 10 μ M lenalidomide, 10 μ M imatinib or with CD40L stimulation. Next, nuclear and cytoplasmic lysates were generated. BID was blotted in the cytoplasmic lysates and TP73 in the nuclear lysates. Actin and a non-specific band were used as loading controls. The western blot shown is representative for N=2. **B.** The ABL1 promoter (NC_000009.12) was downloaded from Ensembl and scanned for consensus sequence TGGGAW using a Python 3.0 script. Arrows indicate the positions of putative Ikaros binding sites. Position 0 is the transcription start site.

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killing, BID is a substrate of Granzyme B²², which could lead to sensitization to T and NK cell-mediated killing³⁰. To address this notion, we pretreated CLL cells with lenalidomide before coculturing them with healthy donor peripheral blood mononuclear cells (HD PBMCs). Cell viability after pretreatment was the same in treated and untreated cells (data not shown). To induce antibody-dependent cell-mediated cytotoxicity (ADCC), these cocultures were performed in the presence of one of two CD20 antibodies (rituximab and ofatumumab) or with an CD38 antibody (daratumumab). Daudi Burkitt lymphoma cells were included as positive controls. A trend towards more killing in lenalidomide pretreated CLL cells was observed, although these results were not significant (Figure 4B). We lastly tested whether lenalidomide sensitized CLL cells to $\gamma\delta$ -T cell mediated killing, as these $\gamma\delta$ -T cell receptor bearing cells can target cells independent of antigen presentation by major-histocompatibility-complex molecules³¹. Killing by $\gamma\delta$ -T cells was not enhanced by lenalidomide pretreatment (Figure 4C).

DISCUSSION

In this study, we found indications that lenalidomide exerts several direct effects on CLL cells, that could contribute to its clinical efficacy. Lenalidomide upregulated BID, p21, and DR6 in CLL cells. Upon CD40 stimulation, both BID²⁶ and p21²⁷ induction are mediated via TP73. Our data indicate that BID upregulation also depends on the ABL-TP73 axis after lenalidomide treatment (Figure 3A). The fact that lenalidomide-mediated BID upregulation was reverted by ABL1 inhibition, suggests that Ikaros proteins could act as repressors for ABL1 transcription, which is relieved upon CRBN-mediated degradation of Ikaros proteins during lenalidomide treatment¹¹⁻¹³. This mechanism of action has also been found during T cell activation, where Ikaros proteins acts as transcriptional repressors of IL-2, which is reverted upon lenalidomide treatment or Ikaros knockdown¹¹. Furthermore validating this repressor model is that fact that the ABL1 promoter contains 9 Ikaros consensus sites (Figure 3B). Considering that the mechanism of action of CD40L-mediated activation of the ABL1/TP73 axis is not known, one might speculate that CD40L could also act via Ikaros.

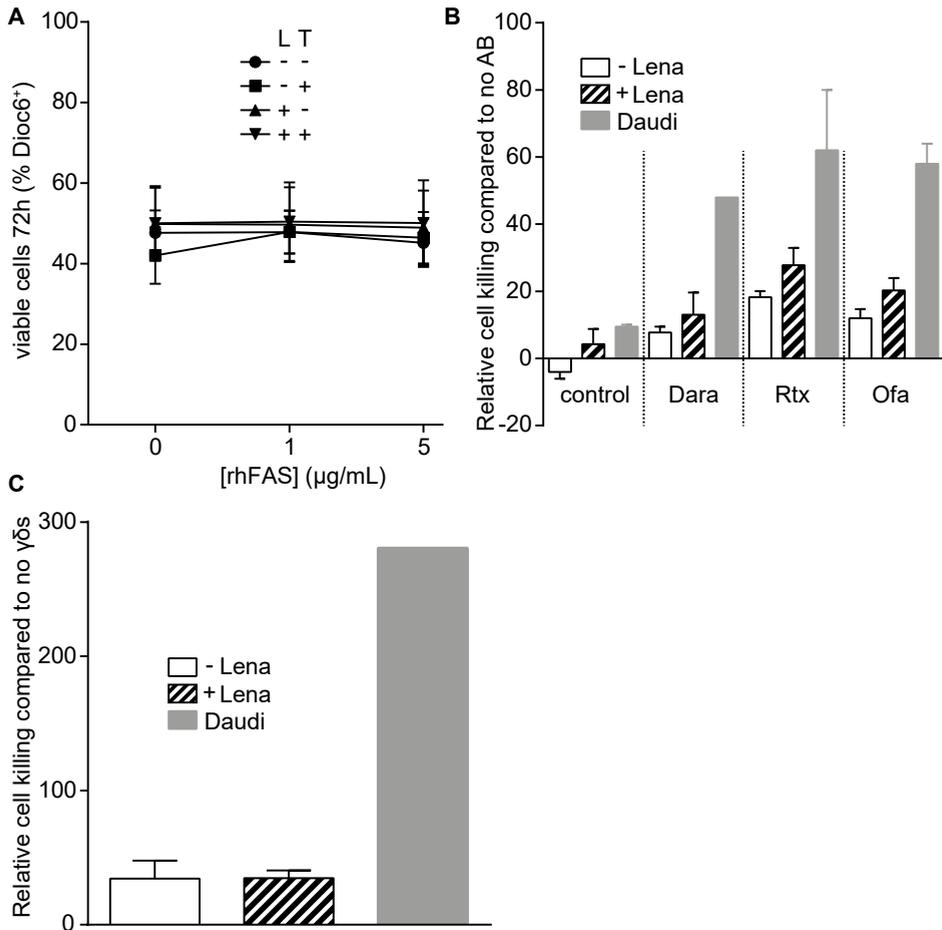


Figure 4: BID upregulation by lenalidomide does not sensitize CLL cells to death receptor or T cell mediated killing. **A.** CLL cells were treated as in Figure 1A with lenalidomide (L) before culturing them with the indicated concentrations of rhFASL, with or without 5 µg/mL rhTRAIL (T) for 48h. Next, viability was determined as in Figure 1A. Points show mean ± s.e.m. for N=3 CLL samples. **B.** CLL cells were treated with 3 µM lenalidomide before adding PBMCs (30:1) in the presence or absence of a control antibody, CD38 antibody daratumumab, (Dara), or CD20 antibodies rituximab (Rtx), or ofatumumab (Ofa). Using cell tracing, the percentage of dead target cells after 3h was determined using propidium iodide staining by flow cytometry and the percentage increase of dead cells compared to the no antibody condition was calculated. Bars show mean ± s.e.m. and are representative of 3 experiments with a total of N=11 CLL samples and Daudi control cells. Each condition was measured in duplo. **C.** CLL cells were treated with 3 µM lenalidomide before coating them with aminobisphosphonate. Next, γδ-T cells were added in a 1:1 ratio. Using cell tracing, the percentage of dead target cells after 24h was determined using propidium iodide staining by flow cytometry and the percentage increase of dead cells from γδ-T cell cocultured CLL samples was compared to CLL cells without γδ-T cells. Bars show mean ± s.e.m. for N=3 CLL samples and are representative of 2 experiments. Each condition was measured in duplo.

Functionally, upregulation of BID might lead to increased sensitivity to death receptor signaling or cell-mediated cytotoxicity, as both death receptor-activated caspase 8²⁰ and Granzyme B²² can cleave BID to its apoptosis-inducing tBID form²⁰. We tested these notions in FAS/TRAIL and cytotoxicity assays, but found no increased apoptosis sensitivity (Figure 4A), although a trend was observed for ADCC. The absence of an effect in these assays could be due to the overexpression of Inhibitor of Apoptosis proteins (IAPs) in CLL cells that counteract death receptor signaling^{32,33}. Regarding death receptor-mediated killing, several evasion mechanisms have been described in CLL cells. First, CLL cells express low levels of FAS and TRAIL receptors in resting conditions³⁴. Second, CLL cells might be unable to form a functioning death-inducing signaling complex (DISC) complex³⁵. In this light, the upregulation of FLIP after lenalidomide treatment (Figure 1B), could also prevent DISC formation³⁶. Third, death receptor signaling leads to the concurrent activation of survival-inducing³⁷ factor NF- κ B³⁸. With respect to the ADCC-mediated killing, it is worth noting that we excluded that lenalidomide pretreated CLL cells were desensitized to killing as a result of CD20 downregulation (data not shown), an effect of lenalidomide found in another study³⁹.

The induction of p21 by lenalidomide treatment could lead to inhibition of cell cycle progression, as p21 acts as a cyclin/CDK2 inhibitor¹⁸. Indeed, it was recently found that *in vitro* CLL cell proliferation by CD40L/IL-4 stimulation is reduced by lenalidomide treatment⁸. An open question remains whether the upregulation of p21 is mediated via the upregulation of TP73 after lenalidomide treatment (Figure 3A)²⁷ or if Ikaros is a direct repressor of p21.

DR6, which was also upregulated by lenalidomide, is part of the death-domain containing TNF receptor family members that includes FAS-R and TRAIL-R (DR-4 and DR-5)⁴⁰. Although upregulation of these latter receptors could potentially sensitize to FAS/TRAIL-mediated killing, DR6 is activated by β -amyloid precursor protein independent of FAS/TRAIL and mainly has a role in neuronal development¹⁹. In addition, it functions independent of caspase 8 and thus does not induce BID activation¹⁹. Nonetheless, other groups have found that also TRAIL-R DR5 is upregulated by lenalidomide treatment²⁵, and based on our MLPA dataset which does not include a probe for DR5, we cannot exclude that it is upregulated. In case DR5 is upregulated, this would indicate that although DR5 upregulation would be expected to synergize with BID upregulation in TRAIL-mediated cell killing, these effects are negated by other cellular programs in CLL cells.

Although short-term beneficial effects of lenalidomide treatment on CLL cells are to be expected based on our data, it is not known whether CLL cells develop resistance against lenalidomide, which is also frequently observed during ibrutinib treatment. Outgrowth of CLL clones bearing the C481S mutation during treatment⁴¹ hampers binding of ibrutinib to this highly specific residue and causes treatment resistance. As the binding of lenalidomide to its target CRBN also depends on a single amino acid⁴² and mutations in CRBN can indeed lead to therapy resistance⁴³, it would be important to study whether lenalidomide treatment also leads to treatment resistance.

In conclusion, we have found several indications that the treatment efficacy of lenalidomide might not only depend on its immunomodulatory function, but also on direct effects on CLL cells, of which inhibition of proliferation is probably the most prominent one.

METHODS

CLL cell isolation and CD40L stimulation

Patient material was obtained from CLL patients, after written 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. Peripheral Blood mononuclear cells (PBMCs) of CLL patients were isolated using ficoll (Pharmacia Biotech, Roosendaal, The Netherlands) and stored in liquid nitrogen. Expression of CD5 and CD19 (both Beckton Dickinson Biosciences [BD], San Jose, CA) on leukemic cells was assessed by flow cytometry (FACS Canto, BD) and analyzed with FACSDiva software (BD). All samples contained at least 90% CD5⁺/CD19⁺. For CD40L stimulation, CLL cells were cultured on CD40L overexpressing NIH-3T3 cells or non-transduced control cells, as described previously⁴⁴.

Reagents

The following reagents were used in the experiments: lenalidomide (Selleckchem Houston, TX, USA), imatinib (Cayman Chemical, Ann Arbor, MI, USA), rhFASL (FAS10, recombinantly produced in house), rhTRAIL (recombinantly produced in house), daratumumab (Genmab, Copenhagen, Denmark), rituximab (Genentech, San Francisco, CA, USA), ofatumumab (Genmab)

Multiplex-ligation dependent probe amplification

RT-MLPA was performed as previously described⁴⁵ using the apoptosis C1 kit that includes probes for the following genes: AIF, APAF1, APOLLON, B2M, BAD, BAK, BAX, BCL-2, BCL-G, BCL-RAMBO, BCL-W, BCL-X, BFL-1, BID, BIK, BIM, BMF, BOK, BOO, CIAP1(2x), CIAP2, DR6, FAS, FASL, FLIP, FLIP2, GRZB, GUSB, HAKAKIRI, LIVIN, MAP1, MCL-1, NIAP, NIP3, NIX, NOXA, OMI, p21, PARN, PERFORIN, PUMA, SERPINB9, SMAC, SURVIVIN, XIAP. To visualize differentially expressed genes, the MLPA data were processed in our web-based MLPA plotter application (<https://martijnvanattekum.shinyapps.io/MLPA/>) which fits a linear model on the data using the limma package in R (<https://www.r-project.org/>) and plots using the ggplot2 package extended with plotly (<https://plot.ly/>).

Flow cytometry

CLL cells were treated with 3 μ M lenalidomide or left untreated for 72h and flow cytometry was performed as described previously⁴⁴ using PE-labeled CD40L antibody 340477 from Becton Dickinson (Franklin Lakes, NJ, USA). Cell viability was measured by Dioc6-PI staining as described before⁴⁴.

Western blot

Cytoplasmatic lysates were created using NP-40 lysis buffer (135 mM NaCl, 5 mM EDTA, 1% NP-40, 20 mM Tris-HCl pH 7.4) with added protease inhibitor (Roche, Basel, Switzerland) and phosphatase inhibitor (Roche). Nuclei were isolated by 15 min centrifugation at 13200 RPM (4°C). Nuclei were subsequently lysed using Laemmli buffer (10% Glycerol and 2% SDS in 63 mM Tris-HCl pH 6.8) with protease and phosphatase inhibitors and western blot

was performed as described previously⁴⁴ using MCL-1, BCL-X_L, and BID antibody (all from Cell Signaling, Danvers, MA, USA), TP73 antibody (Novus, Littleton, CO, USA), and actin antibody (Santa Cruz, Dallas, TX, USA).

FAS/TRAIL and T cell killing assays

CLL cells were treated with 3 μ M lenalidomide or left untreated for 24h before adding combinations of 0, 1, and 5 μ g/mL FASL and TRAIL. After 48h, cell viability was determined using Dioc6-PI staining as described before⁴⁴. In ADCC assays, CLL cells were pretreated for 72h with 3 μ M lenalidomide. After washing away the lenalidomide, target cells (CLL cells or Daudi cells) were stained with 9H-(1,3-Dichloro-9,9-Dimethylacridin-2-One-7-yl) β -D-Galactopyranoside (DDAO) cell trace dye according to manufacturer's instructions and diluted to 4.0×10^5 cells/mL. These cells were then preincubated for 30min with 20 μ g/mL b12 control antibody, rituximab, ofatumumab, daratumumab, or no antibody before adding freshly isolated PBMC's from healthy volunteers in an effector:target ratio of 30:1. These cells were cocultured for 3h in a cell incubator and cell viability of DDAO⁺ cells was determined using propidium iodide staining by flow cytometry. The relative amount of dead cells compared to the no antibody condition was subsequently calculated. For $\gamma\delta$ -T cell killing assays, monocyte-derived dendritic cell (moDC) feeder layers were generated by differentiating CD14 sorted cells from PBMCs with 1000U/mL GM-CSF (Sanofi, Paris, France) and 20ng/mL IL-4 (R&D Systems). After irradiating moDCs with 50 Gy to stop proliferation, $\gamma\delta$ -T cells were FACS sorted from healthy donor PBMCs using V δ 2 FITC (1:50), and V γ 9 PE (1:50) staining, placed on moDCs and expanded for 14 days in ijssele medium with added 1% human AB serum, 10 U/mL IL-7, and 10ng/mL IL-15. CLL cells were pretreated with lenalidomide as described above and subsequently coated with 10 μ M aminobisphosphonate. Next, expanded $\gamma\delta$ -T cells were added in 1:1 ratio and incubated for 24h and the amount of dead cells was determined as described above.

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SUPPLEMENTARY MATERIALS

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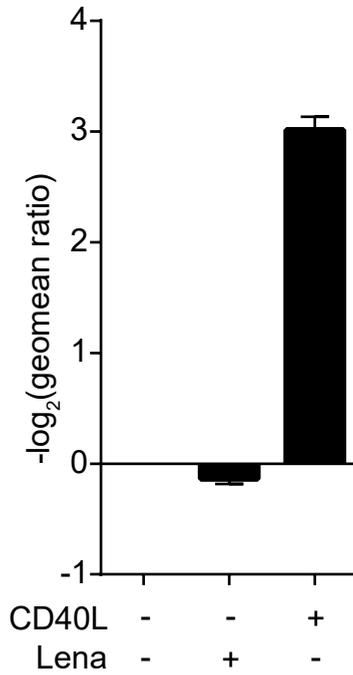


Figure S1: CLL cells (N=2) were cultured as in Figure 1A as indicated and CD40L surface expression levels were determined using flow cytometry. Shown is the log fold ratio of the geometrical mean of signal intensities compared to the unstimulated condition.

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CLL CELLS ACT AS ACTIVE PARTICIPANTS IN MICROENVIRONMENTAL COEVOLUTION

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ABSTRACT

The importance of the tumor microenvironment (TME) in chronic lymphocytic leukemia (CLL) is widely accepted. Yet, the understanding of the complex interplay between the various types of bystander cells and CLL cells is incomplete. Whereas numerous studies have indicated that bystander cells provide CLL-supportive functions, it has only recently become clear that CLL cells actively engage in the shaping of a supportive TME in a process called coevolution. In this review, we describe several recently discovered coevolutionary mechanisms, focusing on the role CLL cells play in A) inducing differentiation and migration of bystander cells, and B) the interactions between bystander cells. Upon T cell interactions, CLL cells secrete cytokines and chemokines such as migratory factors CCL22 and CCL2, which results in further recruitment of T cells but also of monocyte-derived cells (MDCs). Concurrently, CLL-secreted cytokines such as IL-10 suppress cytotoxic T cell functions. With respect to effects on MDCs, CLL cells induce differentiation towards a tumor-supportive M2 phenotype and suppress phagocytosis. Lastly, CLL-associated MDCs contribute to suppression of T cell function by producing immune checkpoint factor PD-L1. Deeper understanding of the active involvement and cross-talk of CLL cells in TME shaping may offer novel clues for designing therapeutic strategies.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a prototypic malignancy that not only depends on intrinsic genetic defects, but is maintained by interaction with bystander cells in microenvironmental niches such as the lymph node (LN). Bystander cells involved include T cells, monocyte-derived cells (MDCs), stromal cells, endothelial cells, fibroblastic reticular cells, and pericytes. Signals emanating from these cells critically affect CLL cells in several key malignancy features such as cell survival, chemo-resistance, cell proliferation, and migration¹. These signals furthermore result in an immune-tolerant milieu in the CLL LN, in which the response to both pathogens² and neo-antigen expressing malignant cells³ is dampened.

Multiple types of signaling molecules are involved in these communication processes: first, interleukins such as interleukin (IL)-4 and IL-21 are involved in cell survival and proliferation^{4,5}. Second, chemokines including CCL2, 3, 4 and 22 have important roles in both the chemo-attraction of cells towards the tumor microenvironment (TME)^{6,7} and tumor cell survival⁸. Third, growth factors such as Insulin-like growth factor 1 can promote survival⁹. Fourth, membrane bound factors from bystander cells such as CD40L and integrins can induce cell survival¹⁰. Fifth, small vesicles such as microvesicles and exosomes containing RNA, proteins, lipids or metabolites that are produced by either bystander cells¹¹ or CLL cells¹² could transmit signals.

Although it is by now well established that the factors secreted by bystander cells are essential for CLL sustenance (summarized in a recent review by Ten Hacken & Burger¹), it has also become clear that these interactions are reciprocal in nature. As shown in other tumor types, upon contact with tumor cells, bystander cells can undergo changes that drive tumor progression, a mechanism dubbed coevolution in accordance with the phenomenon occurring in ecological systems¹³. Considering that CLL bystander cells include immune cells normally involved in highly-adaptable immune responses, they are highly susceptible to (malignant) B cell-derived signals. Next to local changes leading to tumor advance, bystander cell alterations lead to systemic changes that can orchestrate recruitment of peripheral cells towards the TME⁶. Although various studies have suggested that bystander cell changes can take place at the genetic level⁶, recent evidence has shown unaltered stromal genomes, suggesting that microenvironmental signals are not mediated via genetic events⁶. These findings indicate that the stromal alterations are reversible, and that identification of the factors driving stromal cell changes may yield new therapeutic options.

In this review we analyze recent literature and our own recent findings to provide an overview of current evidence that signals emanating from CLL cells are crucial in creating a tumor-supportive TME (Figure 1). Second, as several reports show interdependency of multiple bystander cells, we address how communications among bystanders in the context of CLL can contribute to supportive TME coevolution. We focus on T cells, MDCs and stromal cells that can form a tetrad together with the CLL cell exchanging reciprocal signals. For each of these, the effects of CLL cells towards the bystander cells are discussed, followed by the indirect effects between bystander cells.

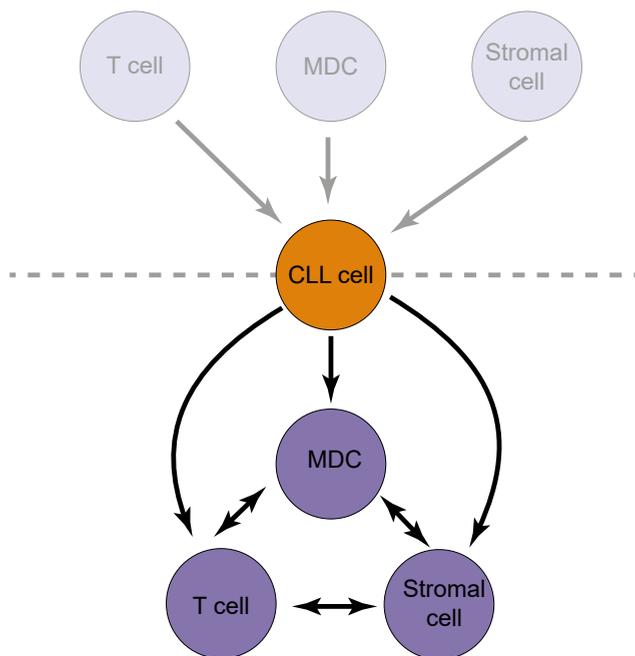


Figure 1: model of interactions in coevolutionary shaping between CLL cells with bystander cells. Although signals emanating from bystander cells such as T cells, monocyte derived cells (MDCs) and stromal cells have been more extensively studied in CLL, the coevolutionary important signals towards and amongst the bystander cells are only recently starting to become elucidated.

T CELL INTERACTIONS

Although it has been described that CD4⁺ Th1 cells recognize CLL antigens³, activated Th1 cells also induce CLL cell proliferation and survival¹⁴. Furthermore, T cells induce a gene expression profile in CLL cells that indicates activation of oxidative phosphorylation in CLL cells¹⁵. With respect to pro-tumor signals from T cells, survival inducing (Interferon(IFN)- γ ¹⁶, IL-4⁴, and CD40L¹⁷) and antigen-independent proliferation factors (CD40L in combination with IL-21⁵) are expressed (Figure 2). These pro-survival signals converge at the upregulation of BCL-2 family members via different signaling cascades, specifically the Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathway¹⁸ and Protein kinase B (AKT) pathway¹⁹. With respect to CLL proliferation, Mitogen-activated protein kinase (MAPK) and Signal Transducer and Activator of Transcription (STAT)3 pathways play additional roles²⁰. In addition, interaction of CLL cells with T cells sensitizes CLL cells to additional pro-tumor signals; first, B cell receptor signaling is enhanced by a microRNA-155-dependent mechanism after CD40L stimulation²¹. Second, CLL cells upregulate adhesion protein CD44 after CD40L stimulation, leading to hyaluronic acid binding, which increases retention in the LN²². Third, next to a direct survival-inducing effect of T cell-secreted IFN- γ on CLL cells, CD38 is upregulated on CLL cells after IFN- γ stimulation. CD38 can subsequently relay MDC-derived CD31 survival signals²³, although this has been difficult to confirm *in vitro*²⁴.

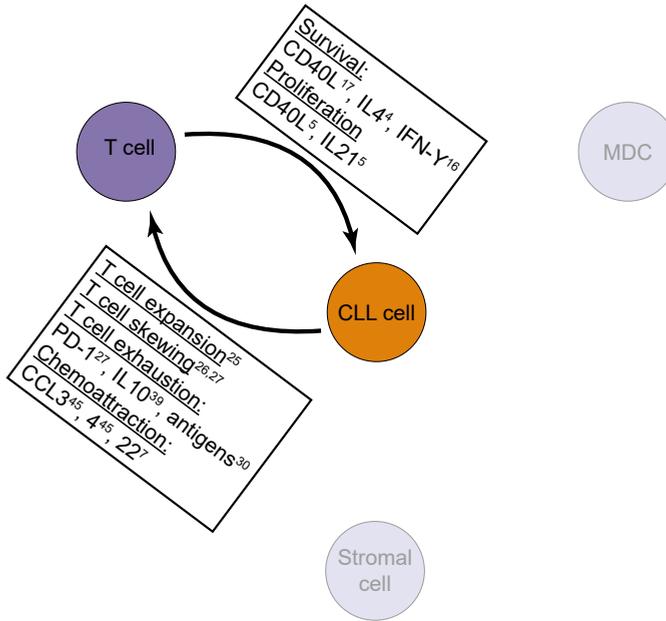


Figure 2: interactions between CLL cells and T cells that contribute to microenvironmental shaping. Within the tetrad of CLL cell, T cell, MDC, and stromal cell, relevant effects (underlined) and signaling molecules involved are indicated.

Functional effects of CLL cells on conventional T cells

Various groups have described aberrations in the T cell population in CLL patients. The total number of both CD4⁺ and CD8⁺ T cells is increased²⁵ and a skewing of their ratio towards CD8⁺ cells occurs in both mouse²⁶ and human²⁷. This skewing does not precede the occurrence of CLL, as it is not present during monoclonal B cell lymphocytosis²⁸, but even at an early disease stage, expansion of the CD8⁺ T cell population is correlated with adverse outcome²⁷. These findings indicate that CLL cells are the causative agent in this correlation. Furthermore, with respect to T cell developmental stages, an increase in effector cells at the expense of naïve cells is observed²⁹. Next to the effects of CLL cells on T cell skewing, CLL cells suppress T cell function³⁰ and suppress effective synapse formation by causing non-polarized release of lytic granules³¹. Lastly, CLL cells are involved in the induction of migration of T cells towards the LN⁷.

Functional effects of CLL cells on non-conventional T cells

The majority of peripheral blood (PB) T cells express the $\alpha\beta$ T cell receptor (TCR), and only 1-10% of CD3⁺ T cells in the PB carry a highly conserved $\gamma\delta$ TCR³². V γ 9V δ 2 T cells form the predominant $\gamma\delta$ T cell subset present in the PB. In contrast to the recognition of peptidic antigens by $\alpha\beta$ T cells, V γ 9V δ 2 T cells respond to stress molecules in malignant cells, in a T cell receptor-dependent yet MHC-independent process. As a consequence, these $\gamma\delta$ -T cells could suppress CLL cells acting independently of MHC antigen presentation³².

Compared to healthy donors however, these $\gamma\delta$ cells show a dysfunctional phenotype in CLL³³. Interestingly, we found that these defects are spontaneously reverted when patient derived $\gamma\delta$ -T cells are cultured in absence of CLL cells³⁴, in support of continuous, active subversion by CLL cells.

As immune suppressive cells, regulatory T cells (T_{reg}) on the other hand secrete several immune suppressive cytokines such as IL-10 and their number correlates with worse prognosis in several tumors³⁵. In CLL, the frequency of Forkhead box protein (FOXP3)⁺ T_{reg} cells is increased in advanced disease³⁶. IL-10 production by T_{reg} cells is higher in the CLL LN than in PB³⁷, in accord with microenvironmental signals engaging in immune-suppressive skewing.

Proposed mechanisms for CLL cell effects on T cells

Functional alterations

Several mechanisms have been linked to the suppression of T cell function by CLL cells. First, CLL cells overexpress immune inhibitory factors such as Programmed death-ligand (PD-L)1 and PD-L2³⁸ and T cells from CLL patients have increased levels of the PD-1 receptor²⁷. In the E μ -T-cell leukemia/lymphoma protein (TCL)1 mouse model, it has been shown that this overexpression is not biased by ageing as adoptive transfer of T cells to these leukemic mice also induces PD-1 on T cells³⁸. Next to PD-1 mediated signaling, CLL cells produce the immune inhibitory cytokine IL-10³⁹. Second, unknown contact-dependent factors produced by CLL cells actively impair the T cell synapse formation⁴⁰, in a mechanism that seems independent of the impaired antigen-presenting capacity of CLL cells themselves. Third, in a mechanism similar to prolonged antigen exposure during chronic infection, T cells in CLL likely adopt an exhausted phenotype due to long-term exposure to unknown antigens expressed by leukemic cells³⁰. This phenotype is characterized by increased expression of exhaustion markers CD160 and CD244³⁰ and an inability to produce adequate levels of immune-activating cytokines upon stimulation⁴¹, similar to the phenotype of T cells directed towards chronic virus infections. Very recently, a link between CLL mediated T cell dysfunction and immune metabolism was suggested by data showing that T cell exhaustion at least partially results from suppression of glucose metabolism⁴². Whether impaired metabolism is a direct consequence of competition for fuels between the tumor cells and T cells as has been shown in experimental models⁴³ or is solely due to CLL-mediated decreased AKT/mTOR signaling⁴² has still to be resolved. It is important to note that the mechanistic causes for T cell expansion and skewing remain largely obscure, but the defects in T cell function might underlie the compensatory expansion seen in CLL patients²⁷.

T cell chemotaxis

Several factors secreted by CLL cells can induce migration of T cells towards the CLL LN. C-C motif chemokine (CCL)22 for instance, is secreted by CLL cells in the LN, which results in the recruitment of T cells⁷. Interestingly, as CCL22 preferentially induces migration of Th2 and T_{reg} CD4⁺ cells⁴⁴, secretion of this chemokine could underlie a skewing in the LN towards CLL-supporting and immune-suppressive T cells at the expense of cytotoxic

T cells. Next to T cell recruitment via CCL22, CLL cells secrete CCL3 and CCL4 upon interaction with MDCs⁴⁵ and levels of CLL3 correlate with increased T cell numbers in *ex vivo* CLL LNs⁴⁶. Finally, the fact that T cells show a reduced motility upon direct contact with CLL cells⁴⁷, could indicate that T cells are retained in the LN once recruited.

MONOCYTE-DERIVED CELL INTERACTIONS

Monocyte-derived cells (MDCs) include monocytes, macrophages, and dendritic cells. These cells can on the one hand secrete essential survival factors for CLL cells, while on the other hand they can potentially mount an immune response against malignant cells as co-stimulators of B or T cell-mediated responses⁴⁸. According to the dichotomized view of macrophage differentiation proposed in normal biology, M1 differentiated immunogenic macrophages mainly convey anti-tumor signals, while M2 wound healing macrophages are pro-tumorigenic overall⁴⁹. This binary differentiation model has however been challenged by others⁵⁰, suggesting that macrophages can exhibit a plethora of other phenotypes, depending on the combined extracellular signals they receive. The delayed disease development associated with MDC depletion in the TCL1 mouse model^{51,52} suggests that MDCs have a crucial, tumor-supportive function in CLL. Their supportive role is furthermore indicated by the observation that a higher number of MDCs correlates with worse prognosis in CLL patients^{53,54}. Whereas MDCs play important roles in CLL cell survival induction⁵⁵ and have migratory effects on CLL cells⁵⁵ (Figure 3), their role in proliferation induction is subordinate; stimulation of CLL cells by macrophages does not induce proliferation (unpublished observation) and furthermore no spatial correlation between MDC-marker CD68 and proliferation marker Ki67 exists in *ex vivo* LNs⁵⁶. Regarding their role in survival induction, we have recently found that MDC-mediated survival depended on chemokine signaling via CCR1¹⁹. Nurse-like cells (NLCs) are monocyte-derived cells which develop following prolonged *in vitro* cultures with CLL blood samples⁵⁵ and these cells have been identified in both the spleen and LNs of CLL patients⁵⁷. NLCs are thought to induce CLL survival effect via factors such as A proliferation inducing ligand (APRIL), B-cell activating factor (BAFF) or CXCL12 (reviewed by Ten Hacken & Burger¹). In line with this, transgenic APRIL overexpression in the TCL1 mouse led to faster disease progression⁵⁸. By contrast, using a novel APRIL-overexpression system and an APRIL decoy receptor, we have recently found *in vitro* that direct effects of APRIL produced by macrophages on CLL cells are negligible⁵⁶. This discrepancy could be reconciled by postulating that *in vivo* effects of APRIL may be indirect, as exemplified by the recent finding that immunosuppressive IL-10 is produced by non-malignant B cells upon stimulation of APRIL receptor TACI⁵⁹⁻⁶¹.

Functional effects of CLL cells on MDCs

In line with the overall pro-tumor effect of CLL-associated MDCs^{51,52}, we⁶² and other groups^{63,64} have found pro-tumor M2 differentiation *ex vivo* and *in vitro* in the presence of CLL cells. Functionally, these cells indeed show impaired immuno-competence, as antigen presentation and immune-response initiation are decreased⁶⁵. In addition, CLL-associated monocytes are defective in their phagocytic function⁶⁶. Moreover, dendritic cells in mice that have undergone adoptive transfer of TCL1 CLL cells show a decrease of MHC class

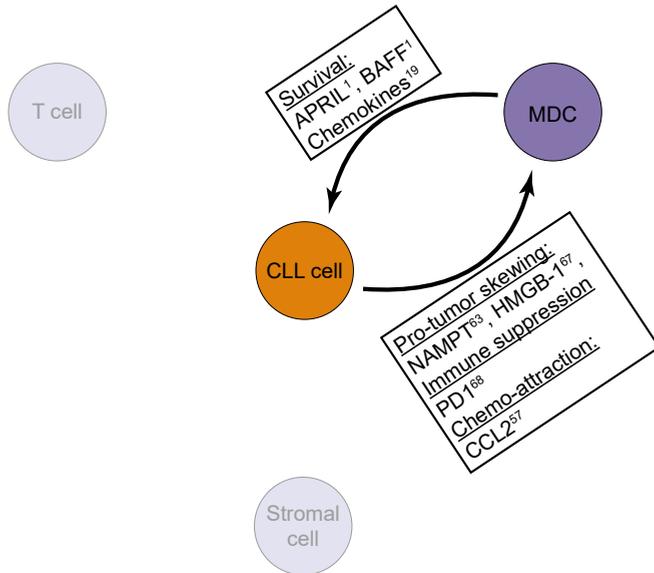


Figure 3: interactions between CLL cells and MDCs that contribute to microenvironmental shaping. Within the tetrad of CLL cell, T cell, MDC, and stromal cell, relevant effects (underlined) and signaling molecules involved are indicated.

IL expression and an increase of the immune suppressive molecule PD-L1⁵¹. We⁵⁶ and others⁵² have found that the CLL LN is interspersed with macrophages. As recruitment of these supportive macrophages depends on chemokine gradients emanating from the LN, it is postulated that CLL cells can provide these migratory signals. Indeed, it has been recently shown that in the TCL1 mouse model, the peritoneal cavity, a reservoir for CLL cells, harbors an increased number of monocytes compared to non-transgenic mice⁵¹.

Proposed mechanisms for CLL cell effects on MDCs

Monocyte polarization

Several CLL-secreted factors have been suggested to contribute to the pro-tumorigenic M2 differentiation of monocytes, which include nicotinamide phosphoribosyltransferase (NAMPT)⁶³ and high mobility group box 1 (HMGB1)⁶⁷. As NAMPT is also secreted by CLL-differentiated MDCs, it could form a positive feedback loop keeping MDCs in a CLL-supportive state⁶³. Besides the potential direct effects of these factors in inducing M2 differentiation, CLL-associated monocytes are primed via an unknown mechanism for M2 differentiation as they show an increased phosphorylation of downstream STAT molecules after stimulation with M2-differentiating cytokines IL-4 and IL-10⁵¹. The persistent M2-differentiating signals emanating from the LN-residing CLL cells in combination with PD-1 stimulation of MDCs, both by CLL cells⁶⁸ and in an autocrine fashion⁵¹, could explain their immune dysfunction. Interestingly, it has recently been shown that this tumor supportive phenotype is reversible, as IFN- γ stimulation results in transdifferentiation of

pro-tumorigenic (M2) CLL-associated monocytes towards M1 macrophages⁶⁹. Similarly, inhibiting PD1 signals could restore macrophage function⁶⁸.

MDC Chemotaxis

Although several chemokines could account for the recruitment of monocytes towards the CLL LN, a critical role for CCR2 has recently been proposed. Adoptive transfer of CLL cells from TCL1 mice to CCR2 knockout mice led to a decrease in monocyte numbers in the spleen. These data are in line with our recent observations using primary human CLL cells, in which we found that inhibition of specifically CCR2 by small molecules could completely abrogate the migration of monocytes towards CLL cells⁶². Others have found that knockout of macrophage migration inhibitory factor (MIF) reduced the number of macrophages in the spleen of TCL1 mice, suggesting an additional role for this chemokine⁷⁰.

STROMAL CELL INTERACTIONS

Stromal cells constitute the connective tissue of organs and supply them with structure, anchoring and supportive signals. By definition, they are of non-hematopoietic origin. Different types of stromal cells include fibroblasts, reticular cells, and endothelial cells. Stromal cells can play a supportive role in various tumor types, including CLL. They were initially described to reside in the CLL bone marrow, but were subsequently identified in almost every organ including the LN⁷¹. Via several mechanisms, stromal cells can directly support CLL cells by increasing their survival⁷² and migration (reviewed by Ten Hacken & Burger¹), but also by inducing CLL cell proliferation⁷³ and by changing CLL cell metabolism⁷⁴ (Figure 4).

Next to these direct effects, stromal cells can govern changes in CLL cells that make them more receptive to other microenvironmental signals. Locally secreted Hypoxia-inducible factor-1 α for instance can induce changes in chemokine receptor expression in CLL cells that consequently retains them in the TME⁷⁵.

Functional effects of CLL cells on stromal cells and mechanisms

As with T cells and MDCs, which types of cytokines are secreted by stromal cells depends on the extracellular signals they receive. In CLL, it has been reported that stromal cells subvert to so-called cancer associated fibroblasts following interaction with malignant cells resulting in the secretion of tumor supportive cytokines⁷⁶. Stromal cells require AKT signaling to support CLL cells⁷⁷. A bidirectional cross-talk in which CLL cells induce AKT and Extracellular signal-regulated kinase (ERK) signaling has been described⁷⁸ and Platelet-derived growth factor is one secreted factors that can cause this activation⁷⁹. Several other groups have pointed at the importance of CLL-secreted exosomes in the differentiation to cancer-associated fibroblasts⁷⁶. One mechanism of action of exosome-mediated differentiation is transfer of microRNA-202-3p, which alters mRNA levels in stromal cells⁸⁰. Also, CLL cells can increase CXCL13 secretion by stromal cells via lymphotoxin- β -receptor activation, which leads to recruitment of CLL cells⁸¹.

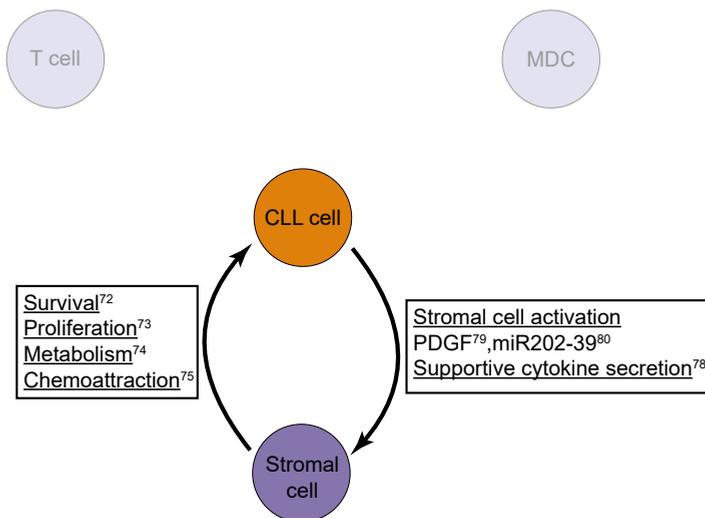


Figure 4: interactions between CLL cells and stromal cells that contribute to microenvironmental shaping. Within the tetrad of CLL cell, T cell, MDC, and stromal cell, relevant effects (underlined) and signaling molecules involved are indicated.

8

INDIRECT EFFECTS

We have so far discussed several direct reciprocal interactions between CLL cells and bystander cells. Considering that all cells within an ecosystem partake in its coevolutionary shaping, interactions between bystander cells can likewise contribute to the formation of a supportive TME in CLL.

Functional effects between monocyte-derived cells and T cells and mechanisms

Based on their role in the normal immune response, it is to be expected that MDCs can also affect the phenotype of T cells in the context of CLL. Indeed, MDCs contribute to T cell skewing in CLL as skewing was reverted after depletion of MDCs via clodronate treatment in the TCL1 mouse model⁵¹ (Figure 5).

MDCs are furthermore involved at several levels of T cell suppression; first, MDCs can induce expression of PD-1 on T cells⁶³, while PD-L1 is upregulated on CLL-differentiated monocytes⁵¹, both contributing to T cell suppression. Second, CLL-differentiated monocytes inhibit T cell proliferation⁶³ and third, they can inhibit T cell activation and promote the differentiation towards T_{reg} cells⁶⁴.

In addition to CLL cells, CLL-differentiated MDCs can secrete chemokines that can attract T cells towards the LN, such as C-X-C motif chemokine (CXCL)12. This chemokine furthermore enhances the expression of CLL cell survival stimuli such as IFN- γ ⁸². Similarly, in mouse studies, splenic monocytes show increased levels of T cell attracting chemokines such as CXCL9 and 10 after adoptive transfer of TCL1 CLL cells⁵¹. Concurrently, expression of the receptor for these chemokines (CXCR3), increases on T cells⁵¹. This indicates that

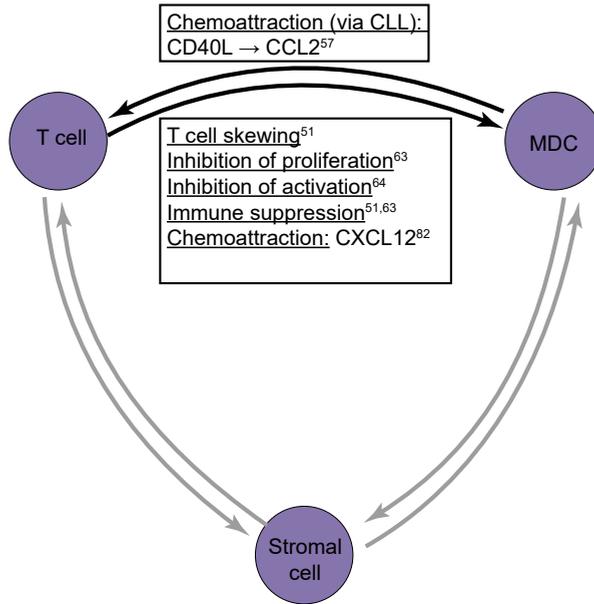


Figure 5: interactions between different bystander cells that contribute to microenvironmental shaping. Within the tetrad of CLL cell, T cell, MDC, and stromal cell, relevant effects (underlined) and signaling molecules involved are indicated.

supporting cells are not only recruited to the TME via induction of attracting chemokines in the LN, but also by an increased susceptibility to recruitment via chemokine receptor upregulation.

A subset of MDCs, the myeloid-derived suppressor cell (MDSC; expressing CD11b and CD33 and low levels of Human Leukocyte Antigen-DR), has recently gained attention in CLL, as it has been shown in other tumor types that these cells are increased in number and can suppress T cell immune responses⁸³. In CLL, an expanded MDSC cell population^{64,84} and suppression of T cells by MDSCs⁶⁴ has been shown. The number of MDSCs furthermore correlated with the number of CLL cells in patients⁸⁴. These data indicate that MDSCs might also suppress the T cell response in the context of CLL.

Differentiation and immune suppressive effects of T cells on MDCs have not been extensively studied in CLL. Regarding migratory effects, we have recently found that stimulation of CLL cells with T cells or T cell factor CD40L induced a strong upregulation of several monocyte-attracting chemokines such as CCL2, 3, 4, 5, 7, 24, CXCL5, 10, and IL-10. We furthermore found that CCL2 can subsequently attract monocytes⁶².

CONCLUSIONS AND OUTLOOK

In parallel with macroenvironments found in nature, coevolution is the driving force in establishing stable supportive interactions between elements within the TME. Considering the data summarized in this review, we here discuss potential consequences for CLL research and therapy.

Open questions: what are the dynamics of coevolution in CLL?

The recent insight in clonal dynamics stemming from Next Generation Sequencing of longitudinal CLL samples may offer some clues into the complex interactions in the TME, although several questions have yet to be answered. First, the change in clone sizes in CLL patients over time regardless of therapy⁸⁵ suggests that natural evolutionary sweeps are taking place. The nature of this important driving force in coevolution is however not known in CLL, but a reasonable assumption is that also here the TME is actively involved. Second, it has been shown in other tumors that different clones of the same tumor can benefit other clones⁸⁶. In that light, the different CLL clones existing in the TME should be considered as separate players (instead of one) when studying coevolutionary shaping. Future research should therefore take into account the interactions between subclones and the effects of the microenvironment on these different clones. Understanding these evolutionary parameters is key to effectively treat CLL, because abolishing one interaction is often insufficient to breach a supportive ecosystem⁸⁷, as adaptations by one of the contributing elements can compensate for the loss of another.

8

Therapeutic consequences

Although knowledge of these compensatory mechanisms would aid to design effective treatment, the potential side-effects that novel therapies have on bystander cells should moreover be considered. Because MDC-mediated antibody responses for instance depend on BTK⁸⁸, ibrutinib treatment reduces FcγR-mediated cytokine production⁸⁸, inhibits activation⁶⁸, and changes metabolism⁶⁸ in monocytes, which can inhibit their immune function. The outgrowth of adoptively transferred CLL cells was however impaired in Btk knockout recipient mice, and macrophages deficient for its upstream kinase Lyn showed diminished CLL-supportive capacity *ex vivo*⁸⁹. This suggests that the effects of ibrutinib on macrophages would be clinically beneficial. The depletion of immune-suppressive MDSCs by ibrutinib⁹⁰ could furthermore support its beneficial clinical effects. Lastly, ibrutinib targets T cell-expressed BTK homolog Interleukin-2-inducible kinase (ITK), which is an important modulator of T cell signaling and function⁹¹. Interestingly, as ITK inhibition preferentially affects Th2 cells because Th1 cells express a compensatory kinase, a potentially beneficial Th1 anti-tumor skewing occurs⁹¹. In the context of CAR-T cell therapy, a T cell expansion and increased tumor clearance was found when concurrently treating with ibrutinib⁹², altogether indicating that ibrutinib treatment can overcome the suppressive effects of CLL cells on T cells. The effects on bystander cells of kinase inhibitor idelalisib are generally CLL-supportive, as idelalisib reduces cytotoxic cytokine production of T cells⁹³ and in macrophages it reduces ADCC⁹⁴ and migration⁹⁵, although inhibition of specifically PI3Ky leads to an immunostimulatory macrophage differentiation⁹⁶. Given the critical pro-tumor effects of bystander cells, these findings suggest that complete tumor eradication after debulking treatment with chemotherapeutics can only be achieved after restoration of T cell function by ibrutinib⁹² or Lenalidomide⁹⁷ that can be complemented with CLL-directed CAR T cells and PD-L1 inhibition⁹⁸. In addition, as ibrutinib treatment results in migration of CLL cells out of the LN, subsequent CLL-attracting chemokine inhibition could avoid (re)formation of a tumor-supportive microenvironment and increase the effectiveness of

cytotoxic therapies. The effectiveness of this migration inhibition approach has for instance been shown *in vivo* in prostate cancer, in which metastases were reduced after CXCR4 inhibition⁹⁹. In conclusion, future insights into the dynamics of coevolution and the effects of (existing) therapies on these dynamics would therefore substantially aid in designing optimal treatment strategies.

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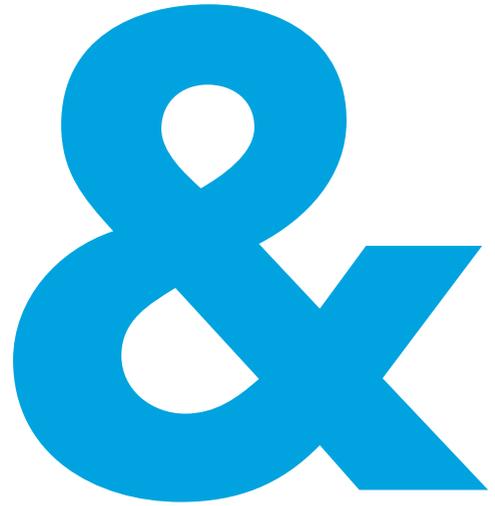
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APPENDICES

LIST OF ABBREVIATIONS

4E-BP	Eukaryotic translation initiation factor 4E-binding protein
ABL1	Abelson murine leukemia viral oncogene homolog 1
ADCC	Antibody-dependent cell-mediated cytotoxicity
AKT	V-Akt Murine Thymoma Viral Oncogene Homolog
APRIL	A Proliferation-Inducing Ligand
ATP	Adenosine triphosphate
A.U.	Arbitrary units
BAFF	B-Cell-Activating Factor
BCL-2	B-Cell Lymphoma 2
BCL-X _L	B-Cell Lymphoma X Large
BCMA	B-cell maturation antigen
BCR	B cell receptor
BFL-1	Pro-survival proteins BCL2-related protein A1
BID	BH3 interacting-domain death agonist
BM	Bone marrow
BTK	Bruton's tyrosine kinase
CCL/CCR	C-C motif chemokine ligand/receptor
CFSE	Carboxyfluorescein succinimidyl ester
CLL	Chronic lymphocytic leukemia
CRBN	Cereblon
CSF-1	Macrophage colony-stimulating factor
CXCL/CXCR	CXC motif chemokine ligand/receptor
DC	Dendritic cell
DDAO	9H-(1,3-Dichloro-9,9-Dimethylacridin-2-One-7-yl) β-D-Galactopyranoside
DEG	Differentially expressed gene
DISC	Death-inducing signaling complex
DR	Death receptor
eIF4	Eukaryotic initiation factor 4
ERK	Extracellular-signal-regulated kinase
FAS	Fas Cell Surface Death Receptor
GEP	Gene expression profile
GSK3	Glycogen Synthase Kinase 3
HD	Healthy donor
HMGB1	High mobility group box 1
IAP	Inhibitor of apoptosis protein
IFN	Interferon
IKZF	Ikaros Family Zinc Finger Protein
IL	Interleukin
JNK	c-Jun N-terminal kinase
JTF	Jurkat-TACI:FAS APRIL reporter cells
Lena	Lenalidomide
LN	Lymph node



MAPK	Mitogen-activated protein kinases
MCL-1	Induced myeloid leukemia cell differentiation protein
MDC	Monocyte-derived cell
MDSC	Myeloid-derived suppressor cell
MLPA	Multiplex ligation-dependent probe amplification
mTOR	Mammalian Target of Rapamycin
Mφ	Macrophage
NADH	Nicotinamide adenine dinucleotide
NAMPT	Nicotinamide phosphoribosyltransferase
NF-κB	Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells
NLC	Nurse-like cells
O/N	Overnight
OXPHOS	Oxidative phosphorylation
PB	Peripheral blood
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PC(A)	Principal component (analysis)
PD-(L)1	Programmed cell death protein 1 (ligand)
PI3K	Phosphoinositide 3-kinase
PIM	Proto-Oncogene Serine/Threonine-Protein Kinase
rh	Recombinant human
S6	Ribosomal Protein S6
SLE	Systemic lupus erythematosus
STAT	Signal Transducer And Activator Of Transcription
SYK	Spleen Tyrosine Kinase
TAC1	Transmembrane activator and CAML interactor
TAM	Tumor-associated macrophage
TCL1	T-cell leukemia/lymphoma protein 1
TCR	T cell receptor
TME	Tumor microenvironment
TNF	Tumor necrosis factor
TRAF	TNF receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TSS	Transcription start site
TWEAK	TNF-related weak inducer of apoptosis
TWEPRIL	TWEAK-APRIL fusion protein
UTR	Untranslated Region

SUMMARY

FORMATION OF A TUMOR-SUPPORTIVE MICROENVIRONMENT IN CHRONIC LYMPHOCYTIC LEUKEMIA:

Addressing the reciprocal interactions in the CLL—T cell—macrophage triad

Chronic lymphocytic leukemia (CLL) is considered a typical malignancy that depends not only on intrinsic genetic defects, but also relies on interactions with bystander cells in the tumor microenvironment (TME). Signals emanating from local bystander cells provide CLL cells with essential survival and proliferation signals. Although it has been established that T cell-mediated effects are largely governed by CD40L, the effects of macrophages are less well-known. Furthermore, the reciprocal nature of the signals between CLL cells, T cells, and macrophages has not been extensively studied. Considering that CLL cells rely on the microenvironment for their survival, these signals would however be amenable to therapeutic targeting. In this thesis we therefore addressed several aspects of CLL-T cell-macrophage interactions. In particular, we have investigated the migration of monocytes towards the microenvironment and the survival effects of macrophages on CLL cells.

CHAPTER 1

In chapter 1 we gave a general introduction to the thesis and introduced the role of the TME in CLL, in order to provide an overview of the scope of the thesis.

CHAPTER 2

In chapter 2, we investigated the differentiation status of lymph node (LN)-residing macrophages. Furthermore we studied whether CLL cells are actively involved in the induction of migration of monocytes towards the LN. In the dichotomized view of macrophage differentiation, tumor associated macrophages (TAMs) are skewed towards either tumor supportive (M2) or immune-surveillance (M1) type. We showed that CLL TAMs were M2 differentiated as a result of factors secreted by CLL cells. Given the presence of macrophages in the LN, we furthermore studied whether CLL cells can recruit monocytes towards the LN, taking into account that potential monocyte-attracting cytokines secreted by CLL cells can be affected by LN-adjacent T cells. We showed that unstimulated CLL cells were unable to recruit monocytes, but that CLL cells stimulated with T cell factor CD40L recruited monocytes via the CCL2-CCR2 axis. In addition, these stimulated CLL cells also secreted several other chemokines such as CCL3, 4, 5, 7, 24, CXCL5, 10, and IL-10.

CHAPTER 3

The exact mechanism of CLL cell survival induction by macrophages is not known, but a significant role has been attributed to the TNFR ligand A Proliferation Inducing Ligand (APRIL). We therefore investigated its role in CLL in chapter 3. We verified that APRIL is expressed by macrophages in the CLL LN and that CLL cells express TACI and BCMA, the receptors for APRIL. Using a novel APRIL overexpressing co-culture system and



recombinant APRIL, we unexpectedly found that APRIL had no effect on CLL cell survival or proliferation, and that it did not activate NF κ B in CLL cells. We then investigated APRIL's contribution in macrophage-mediated CLL cell survival by using an APRIL decoy receptor, TACI-Fc, in macrophage co-cultures. In line with the APRIL overexpression experiments, macrophage-mediated survival did not depend on APRIL.

CHAPTER 4

We discussed the results reported in chapter 3 in the context of current literature on B cell biology in chapter 4. CLL develops from either memory B cells or B1 cells, and B cells in these differentiation stages have been shown to be unresponsive to APRIL signaling. We proposed that the CLL cell has a similar differentiation program as its non-malignant precursor and that it is therefore also not amenable to APRIL signals. Rather, APRIL production leads to production of IL-10 by B10 cells, which can decrease cytotoxicity against CLL cells by causing immune suppression.

CHAPTER 5

In chapter 5, we investigated how macrophages induce CLL cell survival. We first established that macrophage-induced survival depended on the exclusive upregulation of BCL-2 family member MCL-1. Next, we studied which macrophage factor is responsible for this upregulation and via which CLL intracellular pathways it acts. We compared macrophage effects to a known inducer of MCL-1, T cell factor CD40L. Using genome-wide expression profiling of macrophage- and CD40L-stimulated CLL samples, we found activation of the PI3K-AKT-mTOR pathway by both stimuli. Inhibition of this pathway reverted MCL-1 upregulation and CLL cell survival. We furthermore found that the upregulation of MCL-1 depended on formation of the translation initiation complex, and was independent of transcriptional or post-translational regulation. Lastly, we investigated the role of several potential AKT-inducing cytokines, and found that MCL-1 induction and survival critically depended on CCR1, for which the ligand could be secreted by macrophages or in an autocrine fashion.

CHAPTER 6

Malignant cells depend on changes in metabolism for their survival and proliferation. Since the effects of the TME on CLL cell metabolism are currently unknown, we used the gene expression profiles generated for the previous chapter to analyze the effects of both stimuli on oxidative phosphorylation in chapter 6. We found activation of downstream oxphos targets when investigating gene regulation of the MSigDB Hallmark signature. Furthermore, we found upregulation of the functional core multiunit enzymes of the electron transport chain. Moreover, most individual components of these multiunit enzymes were upregulated by both stimuli.

CHAPTER 7

In Chapter 7 we described effects of immunomodulatory drug lenalidomide on CLL cells. This drug is clinically effective in CLL, although it has no cytotoxic effects on CLL cells.

Ikaros, a key protein regulated by lenalidomide, is overexpressed in CLL cells and is critical for B cell development. We therefore investigated potential direct effects of lenalidomide on CLL cells. We found upregulation of BH3-only protein BID, cell-cycle inhibitor p21, and of Death receptor 6 after lenalidomide treatment in a multiplex PCR. The upregulation of BID depended on the ABL1-TP73 axis and the ABL1 promoter contains 9 Ikaros consensus binding sites. Since BID upregulation could sensitize CLL cells to death receptor or T cell-mediated killing, we performed killing assays, but found no sensitization by lenalidomide treatment.

CHAPTER 8

Chapter 8 summarized our data within the context of current literature on coevolutionary signals in the TME. We focused particularly on signals derived from CLL cells. We described that stimulation of CLL cells with T cells leads to the secretion of migration cytokines such as CCL22 and CCL2 that can recruit T cells and macrophages towards the TME. CLL cells furthermore secrete immune-suppressive cytokines such as IL-10 and induce differentiation of macrophages towards a tumor-supportive M2 phenotype via factors such as HMGB-1 and NAMPT. Based on these findings, the chapter closed with considerations for future CLL research and treatment.



NEDERLANDSE SAMENVATTING

DE VORMING VAN EEN TUMOR-ONDERSTEUNEND MICROMILIEU IN CHRONISCH LYMFATISCHE LEUKEMIE:

Een studie naar de wederkerige interacties in de CLL—T cel—macrofaag triade

Chronisch lymfatische leukemie (CLL) wordt gezien als een typische maligniteit die niet alleen afhankelijk is van intrinsieke genetische defecten, maar ook van interacties met zogenaamde omstandercellen in het tumor micromilieu (TMM). Signalen die uitgaan van deze lokale omstandercellen voorzien CLL cellen met essentiële overlevings- en proliferatiesignalen. Hoewel het bekend is dat T cel-gemedieerde overlevingseffecten grotendeels via CD40L plaatsvinden, zijn de effecten van macrofagen grotendeels onbekend. Daarnaast is er weinig bekend over de wederkerige aard van de signalen tussen CLL cellen, T cellen en macrofagen. Aangezien CLL cellen afhankelijk zijn van het micromilieu voor hun overleving, zouden deze signalen echter een startpunt kunnen vormen voor de ontwikkeling van nieuwe therapieën. In dit proefschrift hebben we daarom gekeken naar verschillende aspecten van CLL-T cel-macrofaag interacties. Met name hebben we de migratie van monocytten richting het TMM bekeken en de effecten van macrofagen op CLL cel overleving.



HOOFDSTUK 1

In hoofdstuk 1 hebben we de rol van het TMM in CLL geïntroduceerd, waarna we de strekking van het proefschrift hebben beschreven.

HOOFDSTUK 2

In hoofdstuk 2 hebben we de differentiatie status van macrofagen in de CLL lymfeklier (LK) bekeken. Daarnaast hebben we bestudeerd of CLL cellen betrokken zijn bij het induceren van migratie van monocytten richting de LK. In het dichotome model van macrofaag differentiatie, zijn tumor-geassocieerde macrofagen (TGMs) gedifferentieerd tot een tumor ondersteunende (M2) of immuun (M1) type. We hebben laten zien dat CLL TGMs M2 gedifferentieerd waren door factoren uitgescheiden door CLL cellen. Gezien de aanwezigheid van macrofagen in de LK, hebben we daarnaast bestudeerd of CLL cellen monocytten kunnen rekruteren naar de LK, rekening houdend met het feit dat potentiële macrofaag-aantrekkende cytokines die door CLL cellen gemaakt worden kunnen worden beïnvloed door signalen van naburige T cellen in de LK. We hebben aangetoond dat niet-gestimuleerde CLL cellen niet in staat waren monocytten te rekruteren, maar dat wanneer CLL cellen werden gestimuleerd met T cel factor CD40L, ze monocytten aantrokken via de CCL2-CCR2 as. Daarnaast scheidde deze gestimuleerde CLL cellen verscheidene andere cytokines uit zoals CCL3, 4, 5, 7, 24, CXCL5, 10 en IL-10.

HOOFDSTUK 3

Het exacte mechanisme van macrofaag-geïnduceerde CLL survival is niet bekend, maar een belangrijke rol is toegedicht aan TNFR ligand "A Proliferation Inducing Ligand"

(APRIL). De bijdrage van deze factor hebben we daarom bekeken in hoofdstuk 3. We hebben bevestigd dat APRIL tot expressie komt in macrofagen in de CLL LK en dat CLL cellen TACI en BCMA, de receptoren voor APRIL, tot expressie brengen. Gebruik makend van een nieuw APRIL overexpressie co-kweek systeem en recombinant APRIL, hebben we onverwacht gevonden dat APRIL geen effect had op overleving of proliferatie, en dat het NF- κ B niet activeerde in CLL cellen. Vervolgens hebben we de bijdrage van APRIL aan macrofaag-gemedieerde survival onderzocht door een APRIL decoy receptor, TACI-Fc, toe te passen in macrofaag co-kweken. In overeenstemming met de APRIL overexpressie experimenten vonden we dat macrofaag-gemedieerde survival niet afhankelijk was van APRIL.

HOOFDSTUK 4

Vervolgens hebben we de resultaten uit hoofdstuk 3 bediscussieerd in het kader van de huidige literatuur over B cel biologie in hoofdstuk 4. CLL komt voort uit hetzij geheugen B cellen, hetzij B1 cellen en het is bekend dat B cellen in deze differentiatiestatus niet reageren op APRIL. Daarom stelden wij voor dat de CLL cel een gelijksoortig differentiatieprogramma heeft als zijn niet-maligne precursor en daarom niet reageert op APRIL signalen. APRIL productie leidt daarentegen tot productie van IL-10 door B10 cellen, hetgeen cytotoxiciteit richting CLL cellen kan verminderen via immuunsuppressie.

HOOFDSTUK 5

In hoofdstuk 5 hebben we (andere) factoren onderzocht die konden zorgen voor macrofaag-gemedieerde CLL cel overleving. Ten eerste hebben we vastgesteld dat macrofaag-geïnduceerd overleving afhankelijk was van de exclusieve inductie van BCL-2 familielid MCL-1. Vervolgens hebben we gekeken welke macrofaag factor verantwoordelijk is voor deze inductie en via welke paden deze factor zijn signalen doorgeeft. Daarbij hebben we de effecten van macrofagen vergeleken met die van een bekende regulator van MCL-1, T cel factor CD40L. Gebruik makend van genexpressieprofielen van macrofaag- en CD40L-gestimuleerde CLL cellen, hebben we aangetoond dat het PI3K-AKT-mTOR pad door beide stimuli werd geactiveerd. Remming van dit pad reduceerde de inductie van MCL-1 en CLL cel overleving. Daarnaast hebben we gevonden dat de inductie van MCL-1 afhankelijk was van de formatie van het translatie-initiatie-complex, terwijl deze niet afhing van transcriptionele of post-translationele regulatie. Als laatste hebben we de rol van verschillende AKT-activerende cytokines bekeken. We vonden dat de inductie van MCL-1 en overleving afhankelijk waren van CCR1, een receptor waarvoor macrofagen het ligand produceren.

HOOFDSTUK 6

Maligne cellen zijn afhankelijk van veranderingen in hun metabolisme voor hun overleving en proliferatie. Gezien de effecten van het TMM op CLL cel metabolisme momenteel onbekend zijn, hebben we de genexpressieprofielen uit het voorgaande hoofdstuk gebruikt om de effecten van beide stimuli op oxidatieve fosforylatie (oxfos) te onderzoeken in hoofdstuk 6. We hebben een activatie van downstream oxfos genen gevonden bij het



bekijken van de expressie van een set genen die met oxfos signalering is geassocieerd. Ook hebben we een inductie van de functionele kerncomplex enzymen gevonden van de elektrontransportketen. Daarnaast zagen we dat de meeste individuele componenten van deze complexen ook geïnduceerd werden door beide stimuli.

HOOFDSTUK 7

In hoofdstuk 7 hebben we de effecten van het immunomoduloire geneesmiddel lenalidomide op CLL cellen onderzocht. Transcriptiefactor Ikaros, die wordt afgebroken in cellen na lenalidomide behandeling, komt verhoogd tot expressie in CLL cellen en is cruciaal voor B cel ontwikkeling. Daarom hebben we mogelijke directe effecten van lenalidomide op CLL cellen onderzocht. Daarbij vonden we een inductie van BH3-only eiwit BID, celcyclusremmer p21 en Death receptor 6 na lenalidomide behandeling in een multiplex PCR. De inductie van BID was afhankelijk van de ABL1-TP73 as en de ABL1 promotor bevatte bovendien 9 consensus bindingplekken voor Ikaros. Aangezien BID inductie CLL cellen gevoelig zou kunnen maken voor death receptor- of T cel-gemedieerde celdood, hebben we dit experimenteel onderzocht, maar hebben we geen effect van lenalidomide gevonden.



HOOFDSTUK 8

In hoofdstuk 8 hebben we onze data in de context van de huidige literatuur over co-evolutionaire signalen in het TMM samengevat. Daarbij hebben we vooral aandacht besteed aan de signalen die uitgaan van CLL cellen. We hebben beschreven dat stimulatie van CLL cellen met T cellen leidt tot productie van cytokines zoals CCL22 en CCL2, die T cellen en macrofagen kunnen rekruteren naar het TMM. CLL cellen maken daarnaast immuunsuppressieve cytokines aan zoals IL-10 en induceren differentiatie van macrofagen richting een tumor-ondersteunend M2 fenotype via factoren zoals HMGB-1 en NAMPT. Op basis van deze bevindingen hebben we dit hoofdstuk afgesloten met overwegingen met betrekking tot CLL onderzoek en therapie.

CURRICULUM VITAE

Martijn van Attekum was born 23rd of August 1983 in Maastricht. As he was particularly interested in the mechanisms behind diseases, he started his biomedical studies next to his medicine study at Maastricht University, for which he obtained a B.Sc and M.D. degree respectively. Throughout his education, he developed a keen interest in oncology research and therefore performed several practicals in this field, including a 6 month scientific practical at the Karolinska Institute in Stockholm. After graduation, Martijn worked for a short period as a researcher in the proteomics field at the University Medical Center Utrecht and as a science teacher at finals high school classes. In 2011 he started his PhD in molecular immunohematology under supervision of Prof. E. Eldering and Prof. A.P. Kater.

Because of a growing interest in bio-informatics, he completed a one-year study followed via Coursera in bioinformatics (with distinction) during his PhD that covers the core curriculum of the bioinformatics master program of the University of California San Diego.

Martijn van Attekum is geboren op 23 augustus 1983 te Maastricht. Gezien zijn sterke interesse in de mechanismes achter ziektes, begon hij zijn studie biomedische wetenschappen naast geneeskunde bij de Universiteit Maastricht, die hij afrondde met een B.Sc en M.D. graad respectievelijk. Gedurende zijn opleiding raakte hij steeds meer geïnteresseerd in oncologie onderzoek, waardoor hij meerdere stages (waaronder een stage van 6 maanden bij het Karolinska Instituut in Stockholm) in dit veld heeft gelopen. Na zijn afstuderen heeft Martijn kort als onderzoeker in het proteomics veld bij het Universitair Medisch Centrum Utrecht gewerkt en als scheikunde leraar in eindexamenklassen. In 2011 is hij begonnen aan zijn PhD in moleculaire immunohematologie onder supervisie van Prof. E. Eldering en Prof. A.P. Kater.

Vanwege een groeiende interesse in bio-informatica, heeft hij naast zijn PhD een 1 jaar durende studie gevolgd via het internetplatform Coursera in bioinformatica (met onderscheid afgerond) waarin het kernvak van het bioinformatica master programma van de University of California San Diego aan bod kwam.



PHD PORTFOLIO

Name PhD student: M.H.A. van Attekum

PhD period: 01-09-2011 until 01-09-2016

Name PhD supervisors: A.P. Kater and E. Eldering

1. PhD training

	Year	ECTS
General courses		
AMC world of science	2011	0.7
Lab safety	2011	0.7
Advanced Immunology postgraduate course	2012	1.5
Specific courses		
Oncology Graduate School Amsterdam Apoptosis	2012	1.5
Image analysis in Matlab	2013	1.5
Omics in science	2014	1.5
Bioinformatics and systems biology specialization (core curriculum of the UCSD master program via Coursera)	2015-2016	36.0
Several R courses via Data Camp	2016	2.0
Seminars, workshops and master classes		
Weekly department seminars	2011-2016	4.0
Weekly B cell malignancy consortium seminars	2011-2016	4.0
Bi-weekly oncology seminars	2011-2016	2.0
Master class "High-throughput data analysis in CLL" by Catherine Wu	2016	0.2
(Inter)national conferences		
National		
Dutch Hematology Congress	2012,2014,2015	2.0
Dutch Cancer Foundation (KWF) tumor cell biology meeting	2013,2014,2015	2.0
International		
Young investigators meeting and the International Workshop on CLL, IWCLL (Bonn)	2012,2015	2.0
European Workshop on Cell Death (Monétier-les-Bains, Cyprus, Rome)	2012,2014,2016	4.0
Presentations		
Journal club (10x)	2011-2016	0.5
Department seminar (4x)	2011-2016	0.4
AMC Hematology meeting (3x)	2011-2016	0.4
Oral presentations at Dutch Hematology Congress (2x), KWF meeting (2x), EWCD (2x)	2011-2016	0.6
Poster presentations at IWCLL (2x) and EWCD (1x)	2011-2016	0.8
Retreats		
"Triple I" immunology retreat (co-organizer)	2012,2013	4.0
Oncology Graduate School Amsterdam retreat	2012	0.7

2. Teaching

	Year	ECTS
Supervising		
Supervised Bachelor student	2015	5.0
Supervised Master student	2013	5.0

3. Parameters of Esteem

	Year
Grants	
Research fellowship from MSD (Saal van Zwanenberg)	2008
Selected to participate in Roche continents: "Arts meets science"	2008
Awards and Prizes	
Immunotools award for innovative cytokine research	2013
Poster Prize at the International Workshop on CLL	2014

4. Publications

First authorships

van Attekum MHA, Terpstra S, Reinen E, Kater AP, Eldering E. Macrophage-mediated chronic lymphocytic leukemia cell survival is independent of APRIL signaling. *Cell Death Discovery*. 2016;2:16020.

van Attekum MHA, Terpstra S, Slinger E, von Lindern M, Kater AP, Eldering E. Macrophages confer survival signals via CCR1-dependent translational MCL-1 induction in chronic lymphocytic leukemia. Manuscript accepted in *Oncogene* (2016).

van Attekum MHA, Eldering E, Kater AP. CLL cells act as active participants in microenvironmental coevolution. Manuscript submitted to *Haematologica* (2016).

van Attekum MHA, Lebre MC, Eldering E, Kater AP. CD40 signaling instructs CLL cells to attract monocytes via the CCR2 axis. Manuscript submitted to *Haematologica* (2016).

van Attekum MHA, Kater AP, Eldering E. The APRIL paradox in normal versus malignant B cell biology (news and views). *Cell Death Dis*. 2016 Jun 23;7(6):e2276.

Co-authorships

Pascutti MF, Jak M, Tromp JM, Derks IA, Remmerswaal EB, Thijssen R, **van Attekum MHA**, van Bochove GG, Luijckx DM, Pals ST, van Lier RA, Kater AP, van Oers MH, Eldering E. IL-21 and CD40L signals from autologous T cells can induce antigen-independent proliferation of CLL cells. *Blood*. 2013 Oct 24;122(17):3010.

Wensveen F, Slinger S, **van Attekum MHA**, Brink R, Eldering E. Antigen-affinity controls pre-germinal center B cell selection by promoting Mcl-1 induction through BAFF receptor signaling. *Sci Rep*. 2016 Oct 20;6:35673

Other

Written 2 releases for the public website of our B cell malignancies consortium



DANKWOORD

Wellicht de belangrijkste les geleerd tijdens het promotietraject is dat correlatie niet altijd een oorzakelijk verband betekent. Zou dit wél het geval zijn, zou dat inhouden dat het verrichten van een promotie leidt tot significant haarverlies, of zou de verbouwing van ons personeelsrestaurant ervoor gezorgd hebben dat ik mijn promotie heb kunnen afronden. Maar nee, de werkelijke reden dat ik nu klaar ben, is de hulp en ondersteuning die ik heb gekregen gedurende de promotie. Daarvoor wil ik met name de volgende mensen bedanken:

Promotoren

Eric, jij weet heel goed hoe de wetenschap in elkaar zit. Je kritische manier van denken is erg leerzaam geweest en zal ik in de toekomst waarschijnlijk nog vaak kunnen gebruiken. Ik heb daarnaast veel bewondering voor je schrijfstijl: het in zo weinig mogelijk woorden overbrengen van een duidelijke boodschap is iets waar ik zelf ook altijd naar streef en ik heb er keer op keer van staan kijken hoe je de stukken telkens toch nog compacter wist te krijgen zonder dat de essentie verloren ging.

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&

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CLL patiënten en gezonde donoren

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Familie

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er zelfs een woordenboek aan te pas moest komen. “We gaan voor de gezelligheid” heb ik gelukkig zeer vaak mogen meemaken, waarvoor dank.

Rob, super dat we keer op keer weer mooie gesprekken en interessante filosofische discussies voeren alsof we elkaar bijna dagelijks zien. De terugkerende schaakpotjes hebben mijn analytische vaardigheden zeker verbeterd.

Gijs, hoewel we in een iets andere tak van sport opereren, kan ik je werkhouding altijd erg waarderen. Gedisciplineerd werken is je duidelijk op het lijf geschreven en ik denk dat men daar ver mee kan komen. Ik laat de panja-goede woensdagavondborrels nog even in mijn agenda staan.

Nicky

Jij hebt samen met mij het promotietraject van dichtbij meegemaakt, zowel het onderzoek als de frustraties die daarbij horen. In de afgelopen maanden leek de werkdruk me af en toe boven het hoofd te groeien, maar door jouw geduld en luisterend oor ben ik er toch doorheen gekomen. Hopelijk heb ik je niet te veel heb wakker gehouden met mijn nachtelijke bioritme. Onze alpenvakanties zijn altijd een prachtig avontuur en ik hoop daar nog veel van te mogen meemaken!





2016