Inflammatory cytokines and signaling pathways are associated with survival of primary chronic lymphocytic leukemia cells *in vitro*: a dominant role of CCL2

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ABSTRACT

Background
Chronic lymphocytic leukemia cells show prolonged survival *in vivo*, but rapidly die by spontaneous apoptosis *in vitro*, unless they are co-cultured with stromal cells or non-malignant leukocytes. The objective of this study was to characterize the survival-inducing cross-talk of chronic lymphocytic leukemia cells with their microenvironment to identify novel therapeutic targets.

Design and Methods
We analyzed and compared microarray-based expression profiles of chronic lymphocytic leukemia cells before and after three different survival-inducing culture conditions: (i) stromal cell co-culture, (ii) stromal cell conditioned medium and (iii) high cell density cultures of unsorted peripheral blood mononuclear cells. Cytokine antibody arrays were applied to study the composition of soluble factors present in these cultures.

Results
The different survival-supportive culture conditions induced distinct gene expression changes, the majority of which were common to all three conditions. Pathway analyses identified – in addition to known signaling networks in chronic lymphocytic leukemia – novel pathways, of which Toll-like receptor signaling, nuclear respiratory factor-2 (NRF2)-mediated oxidative stress response, and signaling via triggering receptor expressed on myeloid cells-1 (TREM1) were the most relevant. A high proportion of up-regulated genes were inflammatory cytokines, of which chemokine (C-C motif) ligand 2 (CCL2) was shown to be induced in monocytes by the presence of chronic lymphocytic leukemia cells *in vitro*. In addition, increased serum levels of this chemokine were detected in patients with chronic lymphocytic leukemia.

Conclusions
Our data provide several lines of evidence that an inflammatory microenvironment is induced in survival-supportive cultures of chronic lymphocytic leukemia cells which might be directly or indirectly involved in the prolonged survival of the malignant cells.

Key words: chronic lymphocytic leukemia, cell survival, NRF2, TREM1, CCL2.

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The online version of this article has a Supplementary Appendix.

Introduction

Chronic lymphocytic leukemia (CLL) is a malignancy derived from small mature B cells, which progressively accumulate in the peripheral blood, the lymphoid organs and the bone marrow of patients.[1,2] The vast majority of CLL cells are non-proliferating cells arrested in the G0/G1 phase. It, therefore, appears that prolonged cell survival prevails over increased proliferation in CLL.[3] Proliferating CLL cells are mainly detected in so-called pseudofollicles localized in the lymph nodes and the bone marrow.[4] In these structures, CLL cells are in close contact and appear to interact with $CD4^+$ T cells and $CD14^+$ myeloid cells. There is evidence that the cellular interactions within these pseudofollicles are essential for CLL cell survival and proliferation. The dependence of CLL cells on external factors is further exemplified by the fact that these cells do not proliferate, but die, by spontaneous apoptosis, within 1 week under culture conditions that support the growth of human B-cell lines.[5] On the other hand, CLL cell survival can be maintained *in vitro* by co-culture with mesenchymal stem cells or various stromal cell lines, as well as with dendritic cells and nurse-like cells.[6-10] We recently showed that short-term survival of CLL cells *in vitro* can also be maintained by soluble factors produced by stromal cells, but long-term survival requires direct contact between CLL cells and the stroma in co-cultures.[10] Both, cell surface receptors and extracellular matrix components were described to be responsible for the better survival rates of CLL cells in stromal cell co-cultures.[11-13] There is also evidence that sustained B-cell receptor stimulation increases survival of CLL cells, at least in a subset of patients.[14] The PI3K/AKT, NFκB, MAPK/ERK, WNT and NOTCH signaling pathways have been associated with CLL cell survival.[15] Furthermore, it was shown that consistent and strong expression of anti-apoptotic proteins, such as Bcl-2 and Mcl-1, is a hallmark of CLL.[9,16] All data available so far argue for a complex mechanism ensuring prolonged survival of CLL cells.

In recent years it has become clear that chronic inflammation contributes to cancer progression and even predisposes to different types of cancer. Inflammatory cytokines and chemokines, e.g. interleukin-6 and chemokine (C-C motif) ligand 2 (CCL2), were shown to be associated with tumor progression and metastasis. By acting on survival and differentiation of monocytes to tumor-associated macrophages, these factors generate a tumor-supportive microenvironment.[17]

Immune-related pathways are also known to be of relevance in the development and progression of CLL. Genetic studies have revealed the expression of stereotyped B-cell receptors on CLL cells, and both autoantigens and infectious agents, such as bacteria, are discussed as potential sources of the antigenic stimulation of CLL cells.[14,18-20] In addition, abnormal serum levels of several inflammatory factors have been identified in patients with CLL.

To identify genes and signaling pathways that contribute to the pathogenesis of CLL, we analyzed the transcriptome of CLL cells in three different survival-supportive culture conditions and thereby recognized the importance of inflammatory signaling pathways and cytokines, of which CCL2 was studied in more detail.

Design and Methods

Primary cells and cell lines

Peripheral blood and serum samples were obtained from 52 CLL patients (*Online Supplementary Table S1*) and 25 healthy donors. All patients provided written informed consent, validated by the Ethics Committee from the University of Ulm, in accordance with the Declaration of Helsinki. All CLL cases matched the standard diagnostic criteria for CLL. The human bone marrow-derived stromal cell line HS-5 was purchased from American Type Culture Collection (Manassas, VA, USA).

Cell isolation and culture

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient separation. Thereafter CLL cell preparations consisted of more than 85% $CD5^+CD19^+$ cells as measured by flow cytometry using fluorescein isothiocyanate-conjugated monoclonal antibody with specificity for CD5 (clone L17F12), and allophycocyanin-conjugated monoclonal antibody specific for CD19 (clone HIB19; both antibodies from BD Biosciences, Heidelberg, Germany). Magnetic bead activated cell sorting (MACS) using CD19-MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) was performed to enrich CLL or normal B cells, resulting in greater than 98% purity of $CD19^+$ cells. Peripheral blood monocytes were enriched from blood samples of healthy donors by MACS using CD14-specific MicroBeads and two sequential column purifications. The purities of monocyte preparations were analyzed by flow cytometry using fluorescein isothiocyanate-conjugated monoclonal antibody with specificity for CD14 (clone M5E2), and were higher than 80%.

For microarray-based expression profiling, 3×10^6 PBMC from CLL patients were seeded either on semi-confluent layers of 3×10^6 pre-seeded HS-5 cells or in 15 mL conditioned medium of HS-5 cells in T-75 culture flasks. For high cell density cultures 1×10^7 CLL PBMC were seeded in 4 mL of complete medium per well in six-well plates. Cells were harvested after 1, 2 or 3 days of culture. All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 4 mM L-glutamine, 4.5 g/L glucose, 100 U/mL penicillin, and 100 μg/mL streptomycin (Complete medium), and were cultured at 37°C in a 10% CO_2 humidified incubator.

Cell viability

Apoptotic cell death was detected by flow cytometry using annexin V-phycoerythrin and 7-amino-actinomycin staining, which was performed as described before.[10] All flow cytometry analyses were carried out using a FACSCalibur or FACSCanto II flow cytometer equipped with CellQuest or FACSDiva software (BD Biosciences).

RNA isolation, amplification and labeling for microarray analysis

Total RNA was isolated from frozen cell pellets by Trizol extraction (Invitrogen, Karlsruhe, Germany) and purified on RNeasy Mini spin columns (Qiagen, Hilden, Germany). The quality of RNA preparations was examined with an Agilent Bioanalyzer using RNA 6000 Nano Chips (Agilent, Waldbronn, Germany). Sample RNA was amplified as described previously.[21] Briefly, 2 μg total RNA were used in first- and second-strand cDNA synthesis. Double-stranded cDNA was extracted and employed for *in vitro* transcription using a RiboMAX Large Scale RNA Production System T7 (Promega, Karlsruhe, Germany) according to the manufacturer's recommendations. Samples were labeled with the cyanine fluorochromes Cy3 and Cy5, and,

after combination of control and sample cDNA, purified on Microcon YM-30 filter columns (Millipore, Schwalbach, Germany). In order to block repetitive sequence elements, 25 μg Cot-1 DNA (Roche Diagnostics, Mannheim, Germany), 5 μg poly-A RNA (Sigma-Aldrich, Munich, Germany) and 7.5 μg yeast tRNA (Sigma-Aldrich) were added to the samples.

Hybridization of oligo-microarrays

A set of 36,196 gene-specific 70-mer oligonucleotides (Human Oligo Set 4.0; Operon, Cologne, Germany) was printed in unicates on glass slides coated with epoxy-silane (Schott Nexterion, Jena, Germany). Hybridization was performed as previously described.[22] Briefly, dye-labeled cDNA (Cy3 or Cy5) of cultured and control cells was mixed with Ultra-Hyb hybridization buffer (Ambion, Austin, USA), agitated for 60 min at 60°C and for 10 min at 70°C and subsequently applied to pre-heated (60°C) microarrays mounted in a GeneTAC Hybridization Station (Genomic Solutions, Ann Arbor, USA). Hybridization reactions were performed for 40 h at 42°C with gentle agitation. Thereafter, arrays were automatically washed four times at 36°C with increasing stringency and finally dried by centrifugation.

Data acquisition of microarray experiments, quality control and statistical analysis

Hybridized microarrays were scanned at 5 μm resolution in a two-color Agilent Scanner G25505B with automatically adjusted photomultiplier tube voltages according to the manufacturer's specification. Raw array data were generated from scanned images using Axon GenePixPro Software (v6.1.0.2). The data were pre-processed, quality controlled and analyzed with our in-house developed ChipYard framework for microarray data analysis (http://www.dkfz.de/genetics/ChipYard/) using R and Bioconductor packages.[23,24] Feature signals had to fulfill the following criteria to be considered for analysis: a signal to background ratio of 1.2 or more in at least one channel; a mean to median spot intensity less than or equal to the 75% quantile plus three times the interquartile range of all features on the array; and a feature replicate standard deviation of 0.25 or less per array. Raw signals were normalized using a variance stabilization algorithm.[25] Probes with more than 40% missing values across all samples were removed. To identify differentially expressed genes, the limma package was applied,[26] which uses an empirical Bayes method to moderate the standard errors of the estimated log-fold changes. Based on BLASTing the probe sequence information against the genome, biological annotations were retrieved from EnsEMBL (version 54, NCBI Build 36 of the human genome reference sequence). Data are available at the NCBI Gene Expression Omnibus (GEO) database, accession number GSE18192.

The largest intra-group differences in expression were obtained in the HS-5 co-culture condition. However, since the harvested suspension cell fractions after co-culture were contaminated with approximately 10% HS-5 stromal cells, the results were corrected by depleting HS-5 specific genes. These genes were identified by comparing expression profiles of CLL PBMC and HS-5 cells, which were not co-cultured, on the same microarray platform (dataset BvsH in GEO database).

Quantitative reverse transcriptase polymerase chain reaction

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed as described previously by using SuperScript II and anchored oligo-d(T)$_{20}$ primer (Invitrogen) to generate cDNA templates, and SYBR Green ROX Mix (Abgene, Epsome, UK) for amplification and quantification in an ABI Prism 7900RT Sequence Detection System (Applied Biosystems, Forster City, USA).[27] The efficiency of the amplification was calculated and relative quantification *versus* non-regulated housekeeping genes was performed as described previously. Duplicate qRT-PCR reactions were carried out for four to eight different CLL samples. The qRT-PCR primer sequences used are shown in *Online Supplementary Table S2*.

Cytokine array

Supernatants of 1×10^7 primary CLL cells, co-cultured for 3 days with 1×10^6 HS-5 cells in 4 mL serum-free medium, and of 1×10^6 HS-5 cells only cultured as control samples, were analyzed with a RayBiotech human cytokine antibody array G series 2000 following the instructions of the manufacturer (RayBiotech, Norcross, GA, USA). Briefly, after blocking the array slides, 100 μL of the collected culture supernatants were incubated on the array for 2 h, washed and incubated with biotin-conjugated primary antibodies, and subsequently with streptavidin-conjugated secondary antibodies. Finally, after washing and drying, the arrays were promptly scanned with an Axon Gene Pix 4000A Microarray Scanner. Data were analyzed using GenePix Pro 6.0 software. Mean values of spotted duplicates were calculated and normalized against mean values of internal positive controls. Fold-change values of cytokine-specific data in comparison to positive controls are given to indicate relative expression levels of cytokines. In addition, differences in cytokine levels between CLL/HS-5 co-cultures and control HS-5 cultures are indicated as fold changes comparing co-culture values *versus* control values. Mean fold-change values obtained in three independently performed experiments were calculated and used to rank the expression of cytokines.

Enzyme-linked immunosorbent assay

For CLL/HS-5 co-cultures, 1×10^6 HS-5 cells and 1×10^7 CLL cells were seeded in 4 mL complete medium in six-well plates and cell culture supernatants were collected after 3 days of culture at 37°C in 10% CO_2. Pure HS-5 cell cultures were established accordingly at the same time. Indicated numbers of sorted CLL cells and monocytes were seeded in 250 μL complete medium in 24-well plates, and cell culture supernatants were collected after 2 days of culture at 37°C in 10% CO_2. The chemokines in cell culture supernatants or diluted peripheral blood serum samples were quantified using human quantikine enzyme-linked immunosorbent assay (ELISA) kits as described by the manufacturer (R&D Systems, Minneapolis, USA). Briefly, ELISA plates were incubated with cell culture supernatant or diluted serum samples followed by incubation with horseradish peroxidase-conjugated polyclonal antibody. After addition of a hydrogen peroxide/chromogen mixture, colorimetric changes were measured at 450 nm.

Results

Differential gene expression in chronic lymphocytic leukemia cells under survival-inducing culture conditions

The survival of primary CLL cells *in vitro* is dependent on microenvironmental factors and has been shown to be maintained in co-cultures with bone marrow-derived stromal cells or their conditioned medium.[10] Interestingly, CLL cell survival can also be maintained by culturing unsorted PBMC (>85% $CD5^+CD19^+$) at densities that allow cell-to-cell contact (Figure 1A). The pro-survival activity in these

cultures is mediated by non-malignant leukocytes, since CD19-sorted CLL cells (>98% $CD5^+CD19^+$) die by spontaneous apoptosis also in high cell density cultures.[27] Survival rates of unsorted CLL cells in high cell density cultures were comparable to those observed when CLL cells were cultured in conditioned medium of HS-5 stromal cells, and slightly weaker than survival rates in HS-5 stromal cell co-cultures.

To identify gene expression changes associated with CLL cell survival, microarray-based transcriptome analyses were performed with CLL cells before and after the three different survival-inducing culture conditions mentioned above and described in detail in the *Design and Methods* section. In CLL/HS-5 co-cultures, a brief decline in CLL cell viability was observed during the first 2 days of culture, with stable survival rates starting on day 3. Therefore, the suspended cells of the co-cultures were harvested after 3 days for microarray analysis. Since survival rates of CLL cells were inferior in HS-5 conditioned medium and high cell density cultures, cells were harvested after 2 and 1 days of culture, respectively. The culture of CLL cells in non-supportive conditions rapidly led to apoptosis of the cells, so control cultures for corresponding durations were not suitable for the analysis. Instead, PBMC freshly isolated from the same patients were deprived of survival stimuli for approximately 1 h by storage in complete medium and used as day 0 control cells. An overview of the microarray analyses performed is shown in Figure 1B. The expression changes observed in four or five CLL samples analyzed within each of the three different culture condition groups were relatively homogeneous. The total numbers of genes with statistically significant differential expression induced in the three culture conditions as well as the overlap between the three datasets are presented in Figure 1C. All microarray data are stored in the NCBI GEO database, accession number GSE18192.

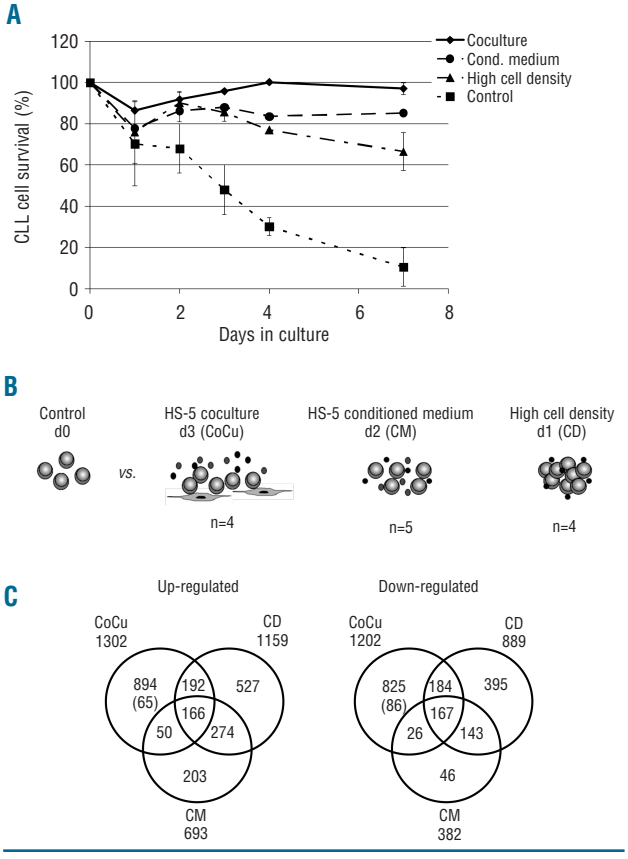

Figure 1. (A) Survival rates of CLL cells *in vitro* under supportive culture conditions. CLL cells were seeded in HS-5 co-cultures, HS-5 conditioned medium and high cell density cultures as described in the *Design and Methods* section. Cell viability was analyzed after annexin V-phycoerythrin – 7-amino-actinomycin D staining by flow cytometry after 1, 2, 3, 4, and 7 days of culture. (n=3) (B) Overview of three data sets acquired by oligo microarray analysis. (C) Summary of up- and down-regulated genes in CLL cells co-cultured with HS-5 stromal cells (Cocu; n=4), their conditioned medium (CM; n=5), or under high cell density condition (CD; n=4). Comparing the three data sets, numbers of genes with differential expression in only one, in two, or in all three conditions were extracted. Results of regulated genes in HS-5 co-culture were corrected by depleting HS-5-specific genes (numbers in parentheses).

Validation of microarray results by quantitive reverse transcriptase polymerase chain reaction analysis using CD19-selected chronic lymphocytic leukemia cells

A randomly selected subset of 23 differentially expressed genes was subsequently analyzed by qRT-PCR in a new set of patients. The results showed that 91% (21 of 23) of the microarray data were verified. Figure 2 summarizes qRT-PCR and microarray results of 14 differentially expressed genes. To evaluate whether the source of the deregulated genes were CLL cells or non-B cells present in the samples, we enriched CLL cells after culture by MACS using CD19-specific MicroBeads to purities of at least 98% $CD5^+CD19^+$ cells. By qRT-PCR analysis, differential expression of 19 out of 21 genes tested was verified with the CD19-sorted cells (Figure 2 shows the results for 14 genes), i.e. the majority of deregulated genes showed differential expression in CLL cells, and only a minor portion was due to non-B cells in the sample. We further investigated the differential expression of several candidate genes at various time points and in all cases observed an early

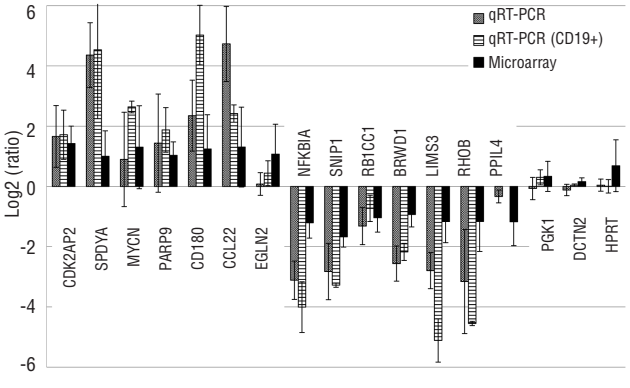

Figure 2. Verification of microarray data by qRT-PCR. qRT-PCR analyses were performed with RNA isolated from CLL cells at day 0 and after 3 days of co-culture with HS-5 cells for a set of selected genes. Equivalent experiments were performed with CLL cell preparations after Ficoll isolation (n=5), as well as after CD19-MACS enrichment (n=3). qRT-PCR results were normalized to three housekeeping genes shown (*PGK1*, *DCTN2*, *HPRT*), and are expressed as $\log_2$ ratios of values at day 3 over day 0. Corresponding microarray data are shown for comparison (n=4). Error bars indicate standard deviations of independently performed experiments.

onset of gene deregulation on day 1 which appeared stable over several days of culture.

A common gene expression pattern is associated with chronic lymphocytic leukemia cell survival in vitro

The three expression profiling datasets were compared and genes with unique regulation in only one of the three culture conditions could be identified (*Online Supplementary Tables S3-S5*). The most striking observation regarding this comparison, however, was that the majority of highly deregulated genes are common to all three culture conditions tested (between 53% and 93% of the top 40 up- and down-regulated genes in each data set; *Online Supplementary Table S6*). This list of genes, which are generally associated with CLL cell survival *in vitro*, consists of 166 up-regulated and 167 down-regulated genes, of which the top candidates are *CCL2*, *CTSL1*, *TPM2*, *RAB13/RAB5B*, and *AL022398.1/TRAF3IP3*, as well as *TSC22D3*, *RGS2*, *DUSP1*, *HBD/HBB*, and *FOSB*, respectively. Results for the top 20 deregulated genes are depicted in Figure 3 and listed in *Online Supplementary Table S7*.

Pathways analysis with these commonly deregulated genes revealed that their major molecular and cellular functions are associated with cell death and cell cycling. Survival of CLL cells *in vitro* is accompanied with gene expression changes in Toll-like receptor signaling, nuclear respiratory factor-2 (NRF2)-mediated oxidative stress response, ATM signaling, triggering receptor expressed on myeloid cells 1 (TREM1) signaling, and p53 signaling. The significance of this analysis and the associated genes within these pathways are listed in Table 1. The most important networks identified in relation to the commonly deregulated genes centered round MYC, p38 MAPK, NFκB, IFNγ, as well as AKT and ERK1/2.

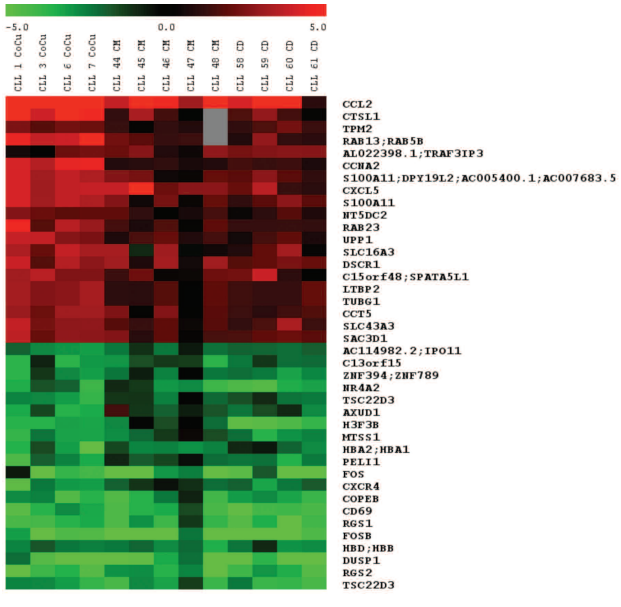

Figure 3. Heat map of the top 20 up- and down-regulated genes that are common to all three survival-inducing culture conditions. Log_2 fold changes of expression in CLL cells after 3 days of HS-5 co-culture (Cocu), 2 days of culture in HS-5 conditioned medium (CM), and 1 day of high cell density culture (CD) are depicted. Red boxes indicate up-regulated genes and green boxes indicate down-regulated genes.

Analysis of cytokine protein levels in co-cultures of chronic lymphocytic leukemia cells and HS-5 stromal cells by antibody arrays

The highly up-regulated genes under survival-inducing culture conditions included many encoding for cytokines and chemokines (e.g. IL6, CCL2, CXCL1, CXCL5). To verify these data and to obtain a general overview of cytokine levels in CLL/HS-5 co-cultures we used a RayBiotech human cytokine antibody array G series 2000. In this way protein levels of 174 different cytokines were independently analyzed in supernatants of CLL/HS-5 co-cultures established from three CLL samples, and the respective pure HS-5 stromal cell control cultures. Results for the top 15 cytokines, according to their mean protein levels in CLL/HS-5 co-cultures, are shown in Table 2. Comparison of CLL/HS-5 co-cultures with pure HS-5 cultures revealed that the majority of highly expressed cytokines are produced by HS-5 stromal cells independently of the presence of CLL cells. Cytokines displaying levels differing by at least 2-fold between the two data sets were CXCL1 (2.43±0.29), CCL4 (100.96±47.40), and

Table 1. Top canonical pathways identified by Ingenuity Pathways Analysis of differentially expressed genes common to all three survival-inducing culture conditions. Calculated *P* values of the analysis, as well as associated genes within the pathways are shown.

Pathway	Genes	*P* value
Toll-like receptor signaling	*CD14, FOS, JUN, TLR1, TLR9*	0.001
NRF2-mediated oxidative stress response	*CBR1, CCT7, EIF2AK3, FOS, GCLM, JUN, JUND, KEAP1*	0.004
ATM signaling	*CDK2, GADD45B, JUN, SMC2*	0.008
TREM1 signaling	*CCL2, CD83, TLR1, TLR9*	0.011
p53 signaling	*BCL2, CDK2, GADD45B, JUN, PMAIP1*	0.015

Table 2. Top 15 cytokines present in CLL/HS-5 co-cultures showing their relative expression level compared to positive controls and their fold expression compared to pure HS-5 cultures.

Cytokine	Relative expression in CLL/HS-5 co-culture	Fold expression compared to HS-5 only culture
IL6	5.98±0.34	1.03±0.06
CXCL1[1]	3.33±0.39	2.43±0.29
CCL4[1]	2.05±0.96	100.96±47.40
TIMP2	1.93±0.43	1.09±0.24
IL8[2]	1.69±0.04	1.17±0.03
CCL2[2]	1.57±0.08	0.92±0.05
MMP1	1.51±0.17	0.83±0.09
MMP3	1.20±0.09	1.17±0.09
CCL7	0.65±0.20	1.21±0.37
CXCL5[2]	0.65±0.04	1.14±0.07
IGF2	0.60±0.02	1.02±0.03
TIMP1	0.47±0.24	1.83±0.95
TGFB1	0.38±0.07	1.19±0.22
CCL3[1]	0.36±0.41	9.18±10.40
LIF	0.25±0.06	1.16±0.26

[1]Cytokines with more than 2-fold higher expression in CLL/HS-5 co-culture compared to pure HS-5 culture; [2]cytokines which were found up-regulated under all three survival-inducing culture conditions by DNA microarrays.

CCL3 (9.18±10.40): CCL4 and CCL3 were not detectable in the control HS-5 stromal cell cultures, but CXCL1 was present in high concentrations in the supernatants of these cultures. Since we detected up-regulation of CXCL1 at the transcriptional level in CLL samples under survival-inducing conditions, this suggests that hematopoietic cells, most likely monocytes, are the source of the additional CXCL1 protein detected in CLL/HS-5 co-cultures. The cytokine array results for all the top 15 cytokines confirmed the transcriptome data obtained, and two of these cytokines, CCL2 and CXCL5, were among those genes which were up-regulated in all three survival-inducing culture conditions tested, showing mean fold-change values at the transcriptional level of 30.44 and 7.04, respectively.

CCL2 is expressed by HS-5 stromal cells and monocytes in co-cultures

Protein expression of CCL2 in CLL/HS-5 co-cultures and pure HS-5 cultures was quantified by ELISA and found to be 10.85±1.53 ng/mL and 8.78±3.33 ng/mL, respectively. To identify the source of CCL2 within the CLL cell sample, qRT-PCR analyses were performed with CD19-sorted CLL cells, CD14-sorted monocytes, as well as unsorted CLL cells before and after co-culture. The purities of the analyzed samples were greater than 98% $CD5^+$ CLL cells after CD19-specific magnetic cell sorting, greater than 90% monocytes after CD14-specific magnetic cell sorting, and greater than 85% $CD5^+CD19^+$ CLL cells for the unsorted samples. As shown in Figure 4A, qRT-PCR analysis confirmed the up-regulation of CCL2 after co-culture in unsorted CLL cells. CCL2 expression was not detectable in CD19-sorted CLL cells. The analysis of sorted monocytes revealed that CCL2 was barely detectable in freshly isolated monocytes, but dramatically induced after 1 day of culture in HS-5 conditioned medium (Figure 4B). Levels of expression were 10- to 30-fold higher in cultured monocytes than in unsorted CLL cells after co-culture. In summary, these data show that CCL2 is not expressed by CLL cells, but that monocytes, which are also present at low percentages in unsorted CLL samples, are the main source of this chemokine.

The presence of chronic lymphocytic leukemia cells induces CCL2 expression in monocytes in vitro

The levels of CCL2 protein in monocyte cultures were quantified by ELISA. These assays revealed increased CCL2 expression in the presence of CD19-sorted CLL cells compared with pure monocyte cultures. CCL2 protein levels correlated with the number of CLL cells added to the monocytes (Figure 4C). These results indicate that CLL cells induce the secretion of CCL2 by monocytes.

The purity of the CLL cell samples used in these experiments was greater than 98% $CD5^+CD19^+$ cells, and no CCL2 could be detected in CLL cell cultures without addition of monocytes (*Online Supplementary Figure S1*), which excludes the possibility that remaining monocytes within the CLL cell preparations were responsible for the increased CCL2 levels.

To investigate whether soluble factors secreted by CLL cells triggered the CCL2 expression in monocytes we seeded CD19-selected CLL cells and CD14-selected monocytes in the upper and lower compartments of transwells with 5 μm pore size (Corning Costar, Lowell, MA, USA). Control cultures without CLL cells, as well as co-cultures of monocytes and CLL cells both added to the lower compartment of the transwells were set up in paral-

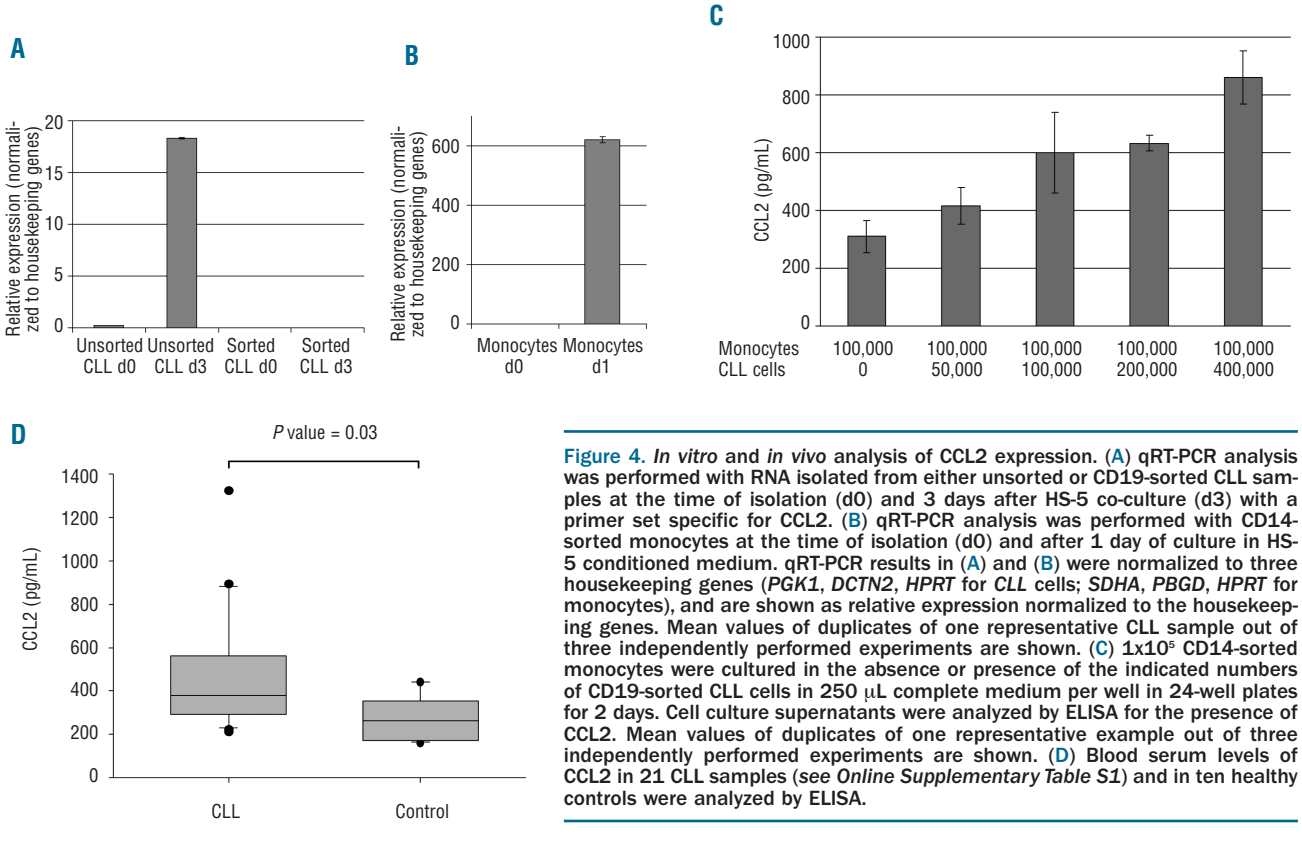

Figure 4. ***In vitro*** **and** ***in vivo*** **analysis of CCL2 expression. (A) qRT-PCR analysis was performed with RNA isolated from either unsorted or CD19-sorted CLL samples at the time of isolation (d0) and 3 days after HS-5 co-culture (d3) with a primer set specific for CCL2. (B) qRT-PCR analysis was performed with CD14-sorted monocytes at the time of isolation (d0) and after 1 day of culture in HS-5 conditioned medium. qRT-PCR results in (A) and (B) were normalized to three housekeeping genes (*PGK1*, *DCTN2*, *HPRT* for *CLL* cells; *SDHA*, *PBGD*, *HPRT* for monocytes), and are shown as relative expression normalized to the housekeeping genes. Mean values of duplicates of one representative CLL sample out of three independently performed experiments are shown. (C) $1x10^5$ CD14-sorted monocytes were cultured in the absence or presence of the indicated numbers of CD19-sorted CLL cells in 250 μL complete medium per well in 24-well plates for 2 days. Cell culture supernatants were analyzed by ELISA for the presence of CCL2. Mean values of duplicates of one representative example out of three independently performed experiments are shown. (D) Blood serum levels of CCL2 in 21 CLL samples (*see Online Supplementary Table S1*) and in ten healthy controls were analyzed by ELISA.**

lel. After 3 days of culture the cell culture supernatants were analyzed by ELISA and showed lower or undetectable levels of CCL2 when monocytes and CLL cells were separated in the transwells, but a strong induction of CCL2 secretion in the direct co-cultures (*data not shown*). These results suggest that CCL2 secretion in monocytes is mediated via direct contact with CLL cells. Future studies will focus on the identification of cell surface receptors involved in this process.

CCL2 has no impact on survival or migration of chronic lymphocytic leukemia cells, but has chemotactic activity for monocytes

A major role of CCL2 is to attract leukocytes to sites of inflammation. We, therefore, tested the chemotactic activity of CCL2 for CLL cells or peripheral blood-derived monocytes isolated by MACS in transwell assays. These experiments clearly showed migration of monocytes towards a stimulus of 10 to 400 ng/mL recombinant human CCL2 or cell culture supernatant of CLL/HS-5 co-cultures, whereas no chemotaxis of CLL cells was observed under the same conditions (*data not shown*). To further test for a potential involvement of CCL2 in CLL cell survival, we added recombinant human CCL2 at concentrations of up to 10 μg/mL to CLL cultures, but did not observe any effect on cell survival rates (*data not shown*). Taken together, these results argue for an indirect effect of CCL2 on the cellular composition of the tumor microenvironment in CLL, by attracting monocytes, which have been shown to induce survival of CLL cells *in vitro*.[27]

CCL2 serum levels are increased in patients with chronic lymphocytic leukemia

To evaluate the potential role of CCL2 in CLL *in vivo*, we quantified peripheral blood serum levels of CCL2 in 21 CLL patients and ten healthy donors by ELISA. We found that the level of CCL2 is significantly higher in CLL patients than in healthy donors (481±272 pg/mL *versus* 274±93 pg/mL; Figure 4D and *Online Supplementary Figure S2*).

Discussion

Understanding the molecular interactions of CLL cells with their microenvironment could considerably help the development of novel targeted therapies. In recent years, several groups have shown that stromal cell co-culture models mimic the *in vivo* situation of CLL cells, providing a good approximation of the *in vivo* microenvironment.[7,10,28,29] We demonstrate that stromal cell co-cultures, stromal cell-derived soluble factors, as well as high cell density cultures of unsorted CLL cells provide survival-inducing conditions with the CLL cells showing distinct as well as overlapping gene expression patterns. Our experimental approach allowed us to identify common events associated with CLL cell survival *in vitro*, and to dissect the complex cellular and molecular interactions into three groups: (i) direct contact of CLL cells with stromal cells, (ii) stromal cell-derived soluble factors, and (iii) cellular interactions as well as soluble factors from CLL cells and non-leukemic autologous leukocytes (Figure 1).

Although we identified specific gene expression changes unique for each condition tested, the majority of highly deregulated genes were common to all three different culture conditions. Thus, our results show that there is a specific expression profile for CLL cells associated with *in vitro* survival. The respective differentially expressed genes are very likely important mediators of CLL cell survival and thus potential targets for novel apoptosis-inducing agents targeting these cells.

In a recent publication, Burger *et al.* analyzed gene expression changes in CLL cells after 14 days of co-culture with nurse-like cells by using HG U133 plus 2.0 oligonucleotide arrays from Affymetrix.[30] When we compared our results with these data, we found a high degree of overlap. The expression of four of the top ten up-regulated genes (*FCRL5*, *TNFRSF17*, *PSAT1*, and *MYCN*) was also increased in our experimental set-up. For CCL3 and CCL4, two T-cell chemokines strongly induced in CLL cells in nurse-like cell co-cultures, we found up-regulation in a subgroup of patients, but we observed no correlation with ZAP70 expression, as described by Burger *et al.* In addition, we found that eight of the top ten down-regulated genes (*HRK*, *RGS2*, *TUBB2A*, *KLF6*, *TSC22D3*, *CHPT1*, *MAFF*, and *RHOB*) were also expressed at reduced levels in two or all three of our culture conditions. Even though a completely different culture condition was used in the study by Burger *et al.*, many genes were regulated similarly as in our experiments, confirming the general importance of these genes in CLL cell survival *in vitro*. Further comparison of our results with data published by Edelmann *et al.*, who co-cultured CLL cells for 2 days with the murine fibroblast cell line M2-10B4 and measured gene expression changes by using Affymetrix U95A microarrays, again revealed a substantial overlap of results.[31] The most significant genes that were up-regulated in both their and our data sets were *CCL2*, *CXCL5*, and *MMP9*, while genes significantly down-regulated in both data sets included *TSC22D3* and *PPP1R15A*. Both the studies by Burger *et al.* and Edelmann *et al.* were performed with co-cultures containing fairly high numbers of CLL cells. The findings described might, therefore, be due to a combination of interactions of CLL cells with the feeder cells, as well as resulting from the high cellular density in these cultures.

Our extensive microarray analysis further identified deregulated genes under survival-inducing conditions that have not been associated with CLL cell survival so far. Pathway analyses using the results of all commonly deregulated genes revealed the relevance of five canonical signaling pathways (Table 1), of which ATM and p53 have been known for many years to be involved in the pathogenesis of CLL.[32] Toll-like receptor signaling was described to have a functional role in CLL cell survival only recently.[33] However, NRF2-mediated oxidative stress response and TREM1 signaling have so far not been associated with CLL or leukemia. NRF2 is a transcription factor regulating antioxidant response to oxidative stress. Impaired NRF2 function leads to Toll-like receptor and nuclear factor-κB activation and can result in cancer, inflammation or other diseases. Since our transcriptome data suggest reduced levels of NRF2 function in CLL cells under survival-inducing culture conditions, this could contribute to CLL cell survival via induction of Toll-like receptor and nuclear factor-κB signaling. Further functional studies will help to explain the role of this pathway in the pathogenesis of CLL.

The TREM1 receptor was identified in 2000 by Bouchon *et al.*, and is known to be associated with innate

immune responses in cooperation with Toll-like receptors and in inflammatory processes.[34] Immune-related and inflammatory conditions have been recognized to be of importance in the pathogenesis of CLL, and anti-inflammatory drugs are currently being tested as potential therapeutic agents for CLL patients.[20] Our data, identifying TREM1 signaling and specific Toll-like receptors, as well as many other factors associated with inflammatory processes, are of importance for further increasing our knowledge on the relation of immunological reactions and CLL development and progression. Future studies targeting the relevant pathways and factors in CLL cells are necessary to develop novel strategies to interfere with both the development and progression of this malignancy.

Since our data revealed an up-regulation of a variety of chemokines and cytokines in CLL samples under survival-inducing culture conditions, we analyzed the composition of soluble proteins in the supernatants of CLL/HS-5 co-cultures by cytokine antibody arrays. A discussion of the most abundant factors identified in these studies is provided in the *Online Supplement* to this article. We focused our analysis on the CCL2 chemokine as one of the most striking proteins with up-regulated expression and secretion under all survival-inducing culture conditions tested. CCL2, which is also known as monocyte chemoattractant protein-1 (MCP-1), is one of the key chemokines that regulate migration and infiltration of monocytes or macrophages. Both CCL2 and its receptor CCR2 have been demonstrated to be involved in various diseases, including cancer and inflammatory disorders.[35] CCL2 is secreted by monocytes/macrophages or dendritic cells in response to infection or inflammation, which leads to the recruitment of more monocytes, whose activation and differentiation were shown to depend on Toll-like receptor signaling.[36] In addition, both TREM1 signaling and NRF2-mediated stress response are pathways interconnected with Toll-like receptor signaling, thereby regulating inflammatory conditions.

The sources of CCL2 in our cell culture models were the HS-5 stromal cells and peripheral blood-derived monocytes. Interestingly, the presence of CLL cells induced expression and secretion of CCL2 in monocytes. Such microenvironment-modulating activity was described for CLL cells also by other groups. Burger *et al.* showed that CLL cells mediate the outgrowth of adherent nurse-like cells from blood-derived monocytes after 2 weeks of culture, and that these nurse-like cells produced survival-inducing factors such as SDF1α, BAFF, and APRIL.[6] Recently, Ding *et al.* observed activation of ERK/AKT signaling in bone marrow stromal cells after contact with CLL cells.[37]

Our functional studies using recombinant CCL2 revealed no direct effect of this chemokine on CLL cell survival or migration, but enhanced migration of monocytes towards CCL2. Since we have previously described pro-survival activity for monocytes in CLL cultures, CCL2 seems to contribute indirectly to a tumor-supportive microenvironment by attracting accessory cells that are able to provide survival signals to the leukemic cells. Targeting CCL2, and thereby the cellular composition of the CLL microenvironment, might therefore be a novel approach to improve treatment options for CLL patients.

The relevance of CCL2 in CLL patients is supported by the fact that serum levels of this chemokine are higher in such patients than in healthy individuals. Higher than normal levels of CCL2 in blood serum have already been described for patients with a variety of tumors, such as acute myeloid leukemia and breast carcinoma.[38,39] In addition, there are studies showing that CCL2 acts as a prominent regulator of prostate cancer growth and metastasis, and targeting CCL2 with inhibiting antibodies induces tumor regression *in vivo*.[40,41]

In summary, our data provide several lines of evidence that an inflammatory microenvironment is induced in survival-maintaining CLL cultures and that this might directly or indirectly support survival of CLL cells. In addition to confirming known signaling pathways, we identified novel candidate genes, in particular CCL2, and pathways, in particular NRF2 and TREM1 signaling. Our data indicate that CCL2, and probably other inflammatory cytokines, are involved in CLL cell survival *in vitro*, and might be of importance *in vivo* as well. More detailed analysis based on our results will help to elucidate the molecular and functional role of these signaling molecules and pathways in the pathogenesis of CLL and to evaluate their potential as novel therapeutic targets.

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