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CCL2 and CXCL2 enhance the survival of primary chronic lymphocytic leukaemia cells *in vitro*.

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Running Title: CCL2 and CXCL2 in CLL

Abstract.

Chronic lymphocytic leukaemia (CLL) is predominantly a disease of accumulation rather than rapid proliferation. To date no cell lines exist as CLL cells undergo rapid apoptosis when cultured *in vitro*, suggesting a favourable *in vivo* microenvironment is required. To identify survival signals we have cultured primary CLL PBMCs at high density, which has previously been shown to dramatically improve survival. Using antibody arrays we measured the level of 42 cytokines in culture supernatants and show that IL-6, IL-8, CXCL2 and CCL2 were highly up-regulated in culture. This is the first report to describe a role for CCL2 and CXCL2 in CLL cell survival. Importantly, CXCL2, IL6 and IL8 were significantly unregulated in primary patient plasma. Addition of either CXCL2 or CCL2 enhanced CLL cell survival while antibodies blocking these chemokines reduced survival. Co-culture of CLL cells and PBMC accessory cells separated by transwells provided a similar degree of survival protection compared to normal culture whereas CLL cells cultured alone died rapidly. Interestingly, CCL2 and CXCL2 appeared to be produced by

CLL cells but only when co-cultured with accessory cells. Thus, we speculate that that accessory cells release soluble factors that promote the production of these pro-survival chemokines from CLL cells and physical interactions were not required. Our data support the concept that the CLL microenvironment is critical and suggests that soluble factors are more important than physical interactions.

Keywords: CLL, CCL2, CXCL2, chemokine, microenvironment

Introduction.

Chronic lymphocytic leukaemia (CLL) is characterised by the progressive accumulation of CD5+ B lymphocytes in the blood. Most of the circulating leukemic cells are arrested in the G0/G1 phase of the cell cycle and the disease is caused by a defect in programmed cell death rather than from rapid proliferation of a malignant clone (1, 2). The clinical course is variable and despite the use of drug combinations such as fludarabine, cyclophosphamide and rituximab (FCR therapy), which have improved overall survival, complete responses remain elusive (3). However, while CLL cells have a long circulating half-life *in vivo* they undergo rapid apoptosis when cultured *in vitro* (4), suggesting that the apoptotic machinery is functional but held in check by interactions with the cellular microenvironment. This has led to the suggestion there is a need to better understand the CLL microenvironment in order to develop more potent or effective therapies (5).

Studies using heavy water suggest that CLL consists of a pool of quiescent cells circulating in the peripheral blood together with a proliferating pool of cells within the bone marrow and lymph nodes (6, 7). This suggests a favourable tumour microenvironment resides in these tissues. Several modulators of CLL cell survival have been identified and include soluble factors and direct cell-cell interactions. For example, IGF-1 (8), IL-4 (9), IL-8 (10), IL-13 (11), CCL3, CCL4 (12), CXCL12, CXCL13, CXCL19 and CXCL20 (13) have all been suggested to act as survival factors or growth promoters in CLL. In addition to cytokines, cell support systems have also been investigated including follicular dendritic cells (14), autologous marrow stromal cells (15, 16), nurse-like cells

(17) and accessory leukocytes (18). Despite the identification of these factors, none have been reported to support survival for longer than 2 weeks, which would allow further insight into the biology and pathogenesis of the disease. Growth in AIM-V media has been reported to improve survival considerable at day 15 compared to RPMI containing 10% serum, although longer periods were not reported (19). Nevertheless, it is more likely that multiple cytokines and cell-cell interactions facilitate CLL cell survival *in vivo* and that a more faithful recapitulation of this environment is required to maintain survival *in vitro*.

Here, we have used recently reported and simplified long-term culture system for primary CLL cells which significantly prolongs their survival *in vitro* and provides a platform to further dissect the complex microenvironmental interactions (20). We measured the levels of 42 different cytokines in primary cultures from CLL patients using a cytokine antibody array and identified two novel chemokines associated with survival. We investigate their ability to modify CLL cell survival and provide evidence that these chemokines are produced by CLL cells, but only in the presence of the accessory cells within the PBMC culture. Interestingly these chemokines do not appear to directly promote CLL survival, indicating a complex network of signals is acting to enhance CLL cell survival. This data further supports the concept that the CLL microenvironment is critical and suggests that soluble factors may be more important than cell-to-cell physical interactions.

Materials and Methods.

Patients: Blood or bone marrow was collected after informed consent from healthy donors or patients with CLL according to protocols approved by the Princess Alexandra Hospital (PAH) Human Ethics committee. Diagnosis of CLL was made according to NCI criteria (21). CLL patients were not undergoing active treatment for their leukaemia or had not received treatment in the month prior to collection.

Cell Purification. Freshly aspirated bone marrow or blood was collected into lithium heparin or EDTA tubes, respectively. Mononuclear cells were isolated by density-gradient centrifugation over

Histopaque-1077 (Sigma-Aldrich, St Louis, MO) and red blood cells were lysed using NH₄Cl ghosting. Normal B cells were purified from healthy donors and CD19 selected CLL Cells were purified from CLL Patients using MACS® cell separation and CD19 Microbeads (Miltenyi Biotec, Germany) according to the manufactures protocol. Additionally, T cells and macrophages were depleted from CLL PBMCs using CD3 and CD14 Microbeads respectively (Miltenyi Biotech) according to manufacturer conditions.

CLL culture and viability. Purified CLL PBMCs were resuspended in RPMI 1640 medium (Gibco Invitrogen, Melbourne, Australia), supplemented with 10% heat inactivated FCS, 100U/ml penicillin G, 100µg/ml streptomycin and 2.92mg/ml glutamine (Gibco; complete media) at a cell concentration of 1 - 60 x 10⁶ cells/ml and a 50% media change was performed weekly, unless otherwise stated. Cell viability was performed at indicated time points by trypan blue exclusion in a Neubeuer counting chamber. Survival was expressed as number of viable cell counts relative to initial plating counts.

Cytokine Array. Human cytokine array was performed using ChemiArray system (Human Cytokine Antibody Array III, Chemicon, Temecula, CA) according to the manufacturers' protocol.

CCL2, CXCL2, IL-6 and IL-8 ELISA. Protein levels of CCL2, CXCL2, IL-6 and IL-8 were measured in the plasma of CLL patients or healthy controls or CLL culture supernatant using enzyme-linked immunosorbent assay (ELISA). Human CCL2/MCP-1 DuoSet ELISA kit was used to detect CCL2 in culture supernatant and Human CCL2/MCP-1 Quantikine ELISA Kit was used to detect plasma CCL2 (both from R&D Systems), Human Growth Regulated Oncogene beta (MIP-2, CXCL2) ELISA Development Kit (Promokine, Germany) was used to detect protein from plasma and culture supernatants, Human IL-6 and Human IL-8 optEIA ELISA kits were used to detect protein from plasma (both from BD), and all were performed according to the manufactures instructions.

In vitro Chemokine Assays. The effect of chemokines IL-6, IL-8, CXCL2 and CCL2 was examined by culturing CLL PBMCs or B cells isolated from healthy donors in the presence of recombinant human IL-6 (2.5ng/ml), recombinant human IL-8 (2.5ng/ml), recombinant human CXCL2

(3.0ng/ml) or recombinant human CCL2 (2.5ng/ml) (all from R&D Systems). The neutralisation effect was examined using monoclonal anti-human CCL2/MCP1 antibody, monoclonal anti-human CXCL1/2/3/GRO pan-specific antibody (R&D Systems) or functional grade purified mouse IgG1 Isotype Control (eBioscience, San Diego, CA), each at a concentration of 1 µg/ml.

Preparation Of Cytological Cells Blocks, Immunofluorescence And Confocal Microscopy. Non-adherent and adherent CLL PBMCs were collected after culturing in the conditions detailed above and made into paraffin embedded cell blocks. Sections were incubated overnight with primary antibodies at 4°C in a humidified chamber. Primary antibodies include mouse anti-human-CD68, rabbit anti-human-CD3 (Dako, Denmark), goat anti-human-CXCL2 (SantaCruz Biotechnology, Santa Cruz, CA) and mouse anti-human-CCL2 (R&D Systems). Following washing, sections were stained with Alexa Fluor® 555 goat-anti-mouse and goat-anti-rabbit and Alexa Fluor® 488 donkey-anti-goat and goat-anti-mouse antibodies. Sections were counterstained with DAPI for nuclear staining before being mounted using Vectashield® (Vector Laboratories, Inc., Burlingame, CA).

Transwell Experiments. Corning Costar 24-well plates with cell-culture insets of 0.4-µm pore diameter (Corning Costar, Cambridge, MA) were supplemented with CD19 selected CLL cells in complete media in the lower chamber. The CD19 negative fraction was prepared in the same medium and was added to the upper chamber. As controls, CD19 selected CLL cells were either co-cultured with the CD19 negative fraction without a porous barrier or cultured alone.

Data Analysis and Statistics. Results are shown as mean ± SEM of at least 3 experiments each performed in duplicate or triplicate. For statistical comparison, the Student unpaired t test was used.

Results

Establishing in vitro Cell Growth of Primary CLL PBMCs.

Increasing Seeding Cell Density Enhances Cell Survival. As published data had suggested soluble factors from other cells within the mononuclear cell population might be important for increased survival of CLL in culture, we decided to culture cells at levels 20-40 times higher than previously published. We reasoned this would more closely mimic the natural situation *in vivo* and increase the

numbers of accessory cells derived from the peripheral blood. For these studies fresh peripheral blood mononuclear cells (PBMC) were harvested from 60 CLL patients (12 females, 48 males, median age of 65 years; range 37-89) (Table 1) with the number of samples used in each experiment indicated in the legend. Initially, PBMC from three patients were Ficoll-purified and cells cultured at concentrations ranging from 1-60 x10⁶ cells/mL in RPMI with 10% FCS and cell survival measured at day 14 using both apoptosis, via PI/Annexin staining and FACS analysis, or trypan blue exclusion. As the results were comparable between each method (data not shown), trypan blue data is shown. We observed a clear relationship between culture cell density and survival, with the highest cell densities (4-6x10⁷/mL) giving 54±5% median survival at day 14 compared to 5±2% for the lowest starting concentration (Figure 1A). Seeding densities below 4x10⁷ cells/ml gave significantly poorer survival (p<0.0001). Previous reports have suggested soluble factors from CLL cells or from accessory cells are in part responsible for the survival of the CLL cell *in vitro* (12, 22). To test this in our system we seeded 5x10⁷ PBMCs/mL and cultured them over 28 days, changing the media at varying intervals from every two days to every two weeks. We found that media changes every two days resulted in a rapid decline in viable cells compared to weekly or fortnightly changes (Figure 1B), despite the high culture density. Additionally, FACS analysis revealed that the longer the cells were cultured *in vitro* resulted in a more enriched population of CD5⁺/19⁺ cells (Figure S1).

Cytokines identified in CLL PBMC cultures. In order to understand what factors influence primary CLL cell survival we attempted to identify the level of 42 cytokines and chemokines produced during culture using a cytokine antibody array (ChemiArray III, Chemicon; outlined in Figure S1). PBMCs were cultured at high density and sampled at week 1 and compared to day 0 complete media. We observed significant increases in the levels of the factors CCL2, CXCL2, IL-8, and IL-6 (Figure 1C). To confirm these findings, culture supernatants from two CLL patients were analysed for CCL2 and CXCL2 protein by ELISA over a 3-week period. Consistent with the cytokine array, high levels of both chemokines were detectable after 7 days in culture and remained high throughout the 3 weeks of culture (Figure 1D and 1E).

Plasma levels of IL-6, IL-8, CCL2 and CXCL2. To determine whether this *in vitro* observation was associated with increased *in vivo* production of these cytokines in CLL patients, we assayed the blood plasma levels for IL-6, IL-8, CCL2 and CXCL2 protein by ELISA and compared the levels to healthy controls. A significant ($p < 0.05$) increase in plasma concentration was observed in CLL patients versus healthy controls for IL-6, IL-8 and CXCL2 (Figure 2A, B and D) although this was not found for CCL2 (Figure 2C).

IL-6, IL-8, CXCL2 and CCL2 enhance CLL Cell Survival in vitro. To confirm that these cytokines had survival-enhancing properties we tested cell survival after 1 week using cells from 5 individual CLL patients, as well as normal B cells, using the established optimal cytokine concentration (previously determined to be 2.5ng/ml for IL-6, IL-8 and CCL2 and 3ng/ml for CXCL2 – data not shown). We observed that the addition of IL-8 and CCL2 resulted in a significant increase in the number of surviving CLL cells at day seven in all patients tested and in 3 out of 5 patients for IL-6 and CXCL2.(Figure 3A and B) but had no effect on growth or survival of normal B cells (Figure 3C). We further tested 2-way and 4-way cytokine combination treatments but found that no combination enhanced survival over the individual treatments alone (Figure 3D).

Both IL-6 and IL-8 have been previously reported to be associated with cell survival or protection against cell death in CLL (10, 23). However, CCL2 and CXCL2 are novel CLL survival factors and therefore we decided to further investigate these chemokines. To determine whether survival was directly due to the addition of these cytokines, functionally blocking antibodies were added to fresh PBMC cultures and survival was analysed after 7 days. As before, the addition of CCL2 and CXCL2 increased cell survival beyond baseline while the subsequent addition of specific CCL2 or CXCL2 blocking antibodies reduced this effect back to baseline survival levels, while non-specific antibodies had no effect (Figure 4A), suggesting blocking these chemokines separately was not critical for survival. However, the addition of both blocking antibodies resulted in a significant reduction in CLL cell survival ($p = 0.02$) (Figure 4B). Analysis of annexin V/PI staining indicated the addition of CXCL2 and CCL2 protected CLL cells from apoptosis. (Figure S2).

CLL PBMCs but not purified CLL cells produce CCL2 and CXCL2 in vitro. To identify if CCL2 and/or CXCL2 was acting in an autocrine or paracrine manner to enhance survival, it was important to determine which cells were responsible for their production. To address this, first we analysed the CCL2 and CXCL2 protein level in CD19-selected CLL cells compared to total CLL PBMCs from the same patient. Higher levels of both CCL2 and CXCL2 protein were observed in CLL PBMCs after one and seven days in culture compared to CD19-selected CLL cells indicating that CLL cells need to be co-cultured for the production of CCL2 and CXCL2 to occur (Figure 4C and D). To confirm this, we next examined cultured CLL PBMCs for the presence of CCL2 and CXCL2 by immunofluorescence. Moreover, to identify which cells were producing the chemokines we co-stained for T cells and macrophages using CD3 and CD68 antibodies respectively. As our cell populations contain less than 10% of non CD19⁺/CD5⁺ cells, as confirmed by FACS analysis, we were able to conclude that the majority of small lymphocytes were CLL cells. We could clearly detect both CCL2 and CXCL2 in the majority of the small lymphocytes with little staining of CD3 and CD68 positive cells (Figure 5A-D). However we were not able to distinguish whether this was due to *de novo* production of these chemokines by CLL cells or whether the chemokines were simply binding to the cell surface. However, given only approximately 25% of CLL cells were positive for the receptors of each (Figure S2) it would suggest CLL cells are producing the chemokines to account for the strong positive staining that was observed. This is in concordance with the previous result where CCL2 and CXCL2 were only produced when CLL cells were co-cultured with accessory cells and indicates that CCL2 and CXCL2 may be produced by CLL cells in the presence of accessory cells or by accessory cells and that they may act in both autocrine and paracrine fashions, respectively.

Accessory cells from PBMCs are critical for CLL cell survival. The reliance on the presence of accessory cells for survival can be clearly demonstrated as culturing pure CLL cells results in a poor survival ($p < 0.001$, $n = 5$) (Figure 6A). The relative abundance of these accessory cells during long-term culture was determined by flow cytometry and morphological analysis of the non-CD5⁺/19⁺ populations. Our results indicated that CD68⁺ cells were rapidly enriched and

differentiated over the first two weeks of culture and remained fairly constant between 30-40% of the non-CLL cells (Figure S3). In contrast to this T cells initially made up the majority of the non-CLL cells on day one but between weeks two and three underwent significant loss, falling from approximately 80% to around 20% of the non-CLL population (Figure S3). In addition, Geimsa stained images of adherent cells after 3 weeks in culture reveals a variety of cell morphologies, which are similar to the nurse-like-cells (NLCs) described by Burger et al (17) (Figure S3). To further dissect which populations are critical in CLL cell survival, the effect of removing specific cell populations was examined. It was observed that the removal of T cells or macrophages, using CD3 or CD14 microbead separation respectively, significantly reduced CLL cell survival (Figure 6B).

Addition of CXCL2 and CCL2 does not rescue purified CLL cells from apoptosis. We also examined whether CXCL2 and CCL2 mediate survival directly via signals to the CLL cells. CXCL2 and CCL2 were added into CD19-selected CLL cultures and CLL PBMCs. Cell survival was examined after 14 days. Despite the significant survival advantage observed for CLL PBMCs cultured with CCL2 or CXCL2 ($p < 0.01$), the addition of either chemokine did not rescue the purified CLL from apoptosis (Figure 6C). A total of 5 patients were examined with the mean % survival shown \pm SEM. This result indicates that CCL2 and CXCL2 do not mediate CLL cells survival directly.

Cell-Cell interactions are not critical for CLL survival in vitro. Finally, to clarify whether direct cell interactions or chemical signals are the crucial element for *in vitro* CLL cell survival, transwell experiments were performed. Both CLL cells co-cultured directly with accessory cells or co-cultured with physical separation transwells provided a similar degree of survival protection while CD19-positive CLL cells cultured alone died rapidly (Figure 6D). These results suggest that direct cell-cell interactions were not critical for CLL cell survival *in vitro* but rather such survival is mediated via soluble factors able to exchange through the transwell membrane.

Discussion.

The tumour microenvironment plays a pivotal role in the survival and persistence of tumour cells *in vivo*. It is believed the microenvironment contributes numerous cellular mechanisms conducive to the survival of many malignancies, including CLL. Recently, emphasis has been placed on identifying and understanding those mechanisms (24, 25). The aim of the present study was to develop a long-term culture system and to identify any novel cytokines or chemokines which influence CLL cell survival *in vitro*. We show here the establishment of long-term cultures of CLL cells in the absence of exogenous stromal cells with significant cell survival at 28 days. Successful culture was highly dependent on initial cell density, infrequent culture media changes, and the presence of accessory cells derived from the patient PBMCs. The starting cell culture density has previously been shown to have a modest effect on survival (26) but maximal cell concentrations used were far lower than those used here ($1 \times 10^6/\text{mL}$). Our data show that increased CLL cell survival correlates with seeding at much higher cell densities (up to $6 \times 10^7/\text{mL}$), a finding confirmed in a recent report (20). Frequent media changes resulted in poorer survival whereas culture in transwells did not alter CLL cell viability. This data therefore supports a model whereby soluble factors rather than cell-cell interactions are critical for survival.

We identified two novel chemokines consistently produced in CLL PBMCs cultures; CCL2 and CXCL2. While chemokines are best known for their chemo-attractant role, they exhibit critical functions in diverse physiological processes including cell death and survival and there is accumulating evidence that chemokines and their receptors play an important role in tumour biology (25, 27). Indeed, CCL2 has been shown to inhibit the apoptosis program induced by growth factor deprivation in T cells (28), protect cardiac myocytes from hypoxia-induced apoptosis (29), and inhibit activation-induced cell death in HIV-infected individuals (30). More recently CCL2 has been implicated in the pathobiology of cancer including carcinoma of the breast, gastro-intestinal tract, pancreas, prostate and myeloma (27). CXCL2 is secreted by monocytes and macrophages and is chemotactic for polymorphonuclear leukocytes and haematopoietic stem cells (31). However, to our knowledge, CXCL2 has not been previously implicated in cancer.

Our results indicate that the addition of exogenous CCL2 and CXCL2 promoted the survival of CLL cells *in vitro*, but combining these chemokines did not further enhance survival. This survival advantage can be abolished by the addition of specific antibodies, however, addition of the antibodies separately does not reduce survival beyond background. Nevertheless, the combined addition of anti-CXCL2 and anti-CCL2 antibodies over a two week period did significantly reduce survival, which indicates that these chemokines may be involved in the same signalling pathway or could be mediating survival via similar mechanisms.

This report also identified IL-6 and IL-8 as survival factors in CLL, which has previously been reported and thus validates our approach (10, 23). Interestingly, IL-6 has been shown to be raised in advanced stages of CLL (32) and it is able to induce the expression of CCL2 in PBMCs (33). We also identified CCL22 (also known as macrophage derived chemokine; MDC) and this is in-keeping with previous reports (34). Conversely, CXCL12 (SDF-1) and IFN- γ was not detected using our cytokine antibody arrays despite previous reports indicating their expression by CLL cells and involvement in CLL cell survival (35-37). These differences may be due to our culturing conditions, including cell density and culture medium, which may affect the types and amounts of cytokines produced. Other T-cell chemokines, such CCL3 and CCL4, implicated in CLL survival (12) were not included in our protein array and thus not detected.

In addition to the chemokines examined in this study, several other chemokines and their receptors have been implicated in CLL cell survival including CXCL13, CXCL20 and CCL19 (38), CXCR4 and CCR7 (39), CXCL13 and CXCR5 (40). Furthermore, adhesion molecules have been found to be imperative in sustaining CLL cell survival including CD49d and CD106 (41). Moreover, it has not been determined whether other extrinsic cytokines or pro-survival signals could act in concert. Nevertheless, it is feasible that multiple cytokines and cell-cell interactions are required to facilitate cell survival. This is in keeping with our results that demonstrate that the addition of CCL2 and CXCL2 into cultures of pure CLL cells does not rescue them from apoptosis. This complex network of signalling events is also reinforced by our findings that CCL2 and CXCL2 are not produced

when cultured as a pure CLL population, but require accessory cells for the production of these chemokines.

It has been reported that CLL cells accumulate in the bone marrow and neoplastic follicles where they receive survival signals from bystander cells (14), while others have shown “nurse-like” cells and stromal cells protect CLL from apoptosis (17). This is in concordance with our findings as CLL cell survival is dramatically reduced when CLL cells are cultured without accessory cells present despite the fact that these accessory cells only contribute a small percentage of the overall culture cell content. Nevertheless, removal of individual T-cell and macrophage populations does not entirely account for the survival signals being provided.

Overall there is a complex picture emerging of CLL cell interactions with accessory cells and cytokine/chemokine networks that may regulate the expression of pro and anti-apoptotic regulators and survival pathways such as PI3 kinase and NFκB. Here we have established a simple culturing method which recapitulates the *in vivo* microenvironment and have identified two novel growth factors, CCL2 and CXCL2, that enhance *in vitro* CLL cell survival predominantly in a paracrine manner and that this survival advantage can be abolished by the use of specific chemokine antibodies. However, the presence of these chemokines alone was not enough to rescue the CLL cells from apoptosis which validates the importance of accessory cells and the supporting microenvironment. This is consistent with the idea that complex chemical signals and/or direct cell-cell interactions occur to facilitate this survival. The mechanisms and signalling pathways that mediate this survival/apoptotic balance still remain to be determined. In addition, a better understanding of these complex interactions could lead to new, more effective treatment strategies for CLL by disrupting the microenvironmental support mechanism.

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Conflict of interest

The authors declare no conflict of interest

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Figure Legends

Figure 1. Identification of culture conditions that enhance long-term CLL cell survival. Freshly isolated mononuclear cells were purified on ficol before culturing in RPMI supplemented with 10% heat inactivated FCS at a cell concentration of $1-60 \times 10^6$ cells/mL. (A) A single patient sample was seeded at the indicated densities and cell survival followed till day 14. (B) PBMCs were culture at 5×10^7 cells/mL for 4 weeks with 50% media changes every 2-3 days, weekly, or fortnightly. (C) CLL PBMCs were cultured in complete media for 7 days before supernatants were incubated with the ChemiArray III membrane. Complete media was used as a negative control to account for non-specific binding. Seven CLL patient samples were tested with similar results. Supernatant was collected from CLL PBMC cultures at indicated time points and CCL2 (D) and CXCL2 (E) protein concentration determined using ELISA. Results are displayed as mean \pm SEM (n=2).

Figure 2. Plasma level of IL-6, IL-8, CCL2 and CXCL2 in CLL Patients. Plasma was collected from CLL patients (n=29 for IL-6, IL-8, CCL2 or n=56 for CXCL2) or normal healthy controls (n=13 for IL-6, IL-8, CCL2 or n=10 for CXCL2) and examined for protein concentration of IL-6 (A), IL-8 (B) CCL2 (C) and CXCL2 (D) level using ELISA.

Figure 3. Effect of IL-6, IL-8, CXCL2 and CCL2 on CLL Cell Survival *in vitro*.

(A) CLL PBMCs were cultured for 7 days either alone or in the presence of recombinant human IL-6 (2.5ng/ml) or IL-8 (2.5ng/ml). Data is displayed as the mean \pm SEM (n=5) and each experiment was performed in triplicate (*p < 0.05 **p < 0.01). (B) CLL PBMCs were cultured with the addition of recombinant human CXCL2 (3.0ng/ml) or CCL2 (2.5ng/ml). Data is displayed as the mean \pm SEM (n=5) and each experiment was performed in triplicate (*p < 0.05 **p < 0.01). (C) B-cells isolated from healthy donors were incubated with CCL2 and CXCL2 as described above. (D) Measurement of cell viability by trypan blue exclusion following the addition of recombinant human IL-6, IL-8, CCL2 or CXCL2 in different combinations. Data is represented as the mean fold-change \pm SEM (n=6, each performed in duplicate or triplicate; *p < 0.05 **p < 0.01).

Figure 4. Mechanism of chemokine effect in CLL *in vitro* culture. (A) CLL PBMCs were cultured in the presence of CCL2 (2.5ng/ml) or CXCL2 (3ng/ml), with or without the addition of anti-human CCL2 antibody, anti-human CXCL1/2/3/GRO pan-specific antibody, and/or an isotype control antibody, all at a concentration of 1 μ g/ml. Data is displayed as the mean \pm SEM and is representative of 1 patient performed in triplicate. (B) Cell viability was examined after 14 days after weekly addition of CXCL2 and/or CCL2 or isotype control antibody (all at 1 μ g/ml). Data is displayed as the mean \pm SEM (n=5 each performed in duplicate or triplicate; *p < 0.05). CLL PBMCs or CD19 selected CLL Cells were examined for CCL2 (C) and CXCL2 protein (D) expression after 1 day and 1 week using ELISA. Data is displayed as mean \pm SEM (n=2, each in triplicate; *p < 0.05 **p < 0.01 ***p < 0.001).

Figure 5. Confocal microscopy detect CCL2 and CXCL2 in cultured CLL PBMCs. Macrophage lineage cells and T cells were examined in 14 day old CLL PBMC cultures for the production of CCL2 and CXCL2 by immunofluorescence staining of CD68 and CD3 (magnification = 63X). CD68 positive macrophage cells were examined for CXCL2 and CCL2 (A) and (B) respectively and CD3 positive T cells cells were examined for CXCL2 and CCL2 (C) and (D) respectively. Cells stained positively for CD68 and CD3 appeared red, cells stained positively for CCL2 and CXCL2 appear green and nuclear staining appeared blue (DAPI).

Figure 6. Accessory cells protect CLL cells from apoptosis *in vitro*. (A) CLL PBMCs or CD19 purified CLL cells were cultured as described previously and cell viability determined after 14 days. Data is displayed at mean \pm SEM (n=5, each performed in duplicate or triplicate; ***p < 0.001). (B) Cell viability was examined after 14 days on cultured CLL PBMCs or indicated cell subsets. The data shown mean \pm SEM (n=5 each performed in duplicate or triplicate; *p < 0.05 **p < 0.01*** p < 0.001). (C) CLL PBMCs or CD19 selected CLL Cells were cultured in the presence of recombinant human CXCL2 (2.5ng/ml) or CCL2 (2.5ng/ml) and cell viability determined after 14 days. The data shown is mean \pm SEM (n=4; *p < 0.05). (D) CD19 selected cells were cultured either alone or in the presence of accessory cells (either co-cultured or separated by a transwell) and cell viability determined after 14 days using trypan blue exclusion. Data is displayed as the mean \pm SEM (n=3) and each experiment was performed in triplicate (**p < 0.01*** p < 0.001).

Tables.

Table 1. Molecular and clinical features of patients.

Characteristic

N	60
Sex [n(%)]	
Male	48 (80%)
Female	12 (20%)
Age	
Yr, median(range)	64 (37-89)
Rai [n(%)]	
0	27 (45%)
1-2	25 (42%)
3-4	8 (13%)
Binet [n(%)]	
A	41 (68%)
B	12 (20%)
C	7 (12%)
Lymphocytes (x10⁹/L) median (range)	28.8 (2.9-176.5)
CD19+/CD5+ (%) median(range)	80 (23%-98%)
CD38 [n(%)]	
Positive	22 (37%)
Negative	38 (63%)
Zap70 [n(%)]	
Positive	36 (60%)
Negative	15 (25%)
Not assessed	9 (15%)
FISH	
17p deletion	2 (3%)
11q deletion	6 (10%)
Trisomy 12	5 (8%)
Normal karyotype	27 (45%)
13q deletion	20 (33%)

