CD62L as a therapeutic target in chronic lymphocytic leukemia

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Running Title. CD62L in CLL survival.

Keywords. CLL, CD62L, in vitro survival

Financial Support: This work was funded by grants from the Australian Leukaemia Research Foundation (DG and NM), Uniqest (NM) and the Princess Alexandra Hospital Cancer Collaborative Group (DG, PM and NM). UQDI gratefully acknowledges all those patients who donated tissue for this project. NS is supported by a Cancer Council Queensland Senior Research Fellowship.
Abstract

**Purpose:** Despite advances in the treatment of chronic lymphocytic leukemia (CLL), the disease remains incurable with standard therapies and relapse is inevitable. A growing body of evidence indicates that alterations in the adhesion properties of neoplastic cells play a pivotal role in the development and progression of CLL.

**Experimental Design:** The expression of 71 cell surface molecules was examined on CLL PBMCs over 3 weeks in culture. The most highly up-regulated marker, CD62L, was examined further for expression on CD5+/CD19+ CLL cells *in vitro* and in lymph node and bone marrow biopsies. The pro-survival role of CD62L was examined using a functional blocking antibody and therapeutic potential evaluated by comparison with current chemotherapy agents.

**Results:** Blocking CD62L resulted in apoptosis of CLL cells but not peripheral blood mononuclear cells (PBMCs) from healthy donors suggesting a novel role for CD62L in CLL cell survival. The beneficial effect of co-culturing CLL cells with bone marrow stromal cells or endothelial cells does not protect CLL cells from anti-CD62L related toxicity. Moreover, combining fludarabine or mafosfamide with the anti-CD62L *in vitro* produced an additive effect both with and without stromal cells.

**Conclusion:** This is the first reported data showing that blocking the activation and homing marker, CD62L, regulates CLL cell survival *in vitro*. These data also suggest that therapeutic antibodies against CD62L may provide additional clinical benefit to CLL patients receiving current standard chemotherapy protocols.
Translational Significance

Although the management of patients with chronic lymphocytic leukaemia (CLL) has improved over the past decade it still remains an incurable disease. Contributing to the poor outcome is a lack of knowledge of the underlying pathobiology of CLL. High levels of CD62L have previously been shown to contribute to the homing and retention of lymphocytes to the bone marrow and lymph nodes. In this study, we show, for the first time, that the expression of CD62L also activates a novel prosurvival signal in CLL cells. Significantly, inhibition of CD62L using therapeutic antibodies induces apoptosis equivalent to that seen with current chemotherapeutics and antagonises stromal-induced survival. Thus, we have identified a novel prosurvival signal in CLL cells that when antagonised can induce cell death equivalent to current CLL chemotherapeutics and when combined with current chemotherapeutic cocktails can add significantly to the cytotoxic response.
Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults in Western society and is characterised by a progressive accumulation of monoclonal CD5+/CD19+ B lymphocytes in the peripheral blood, bone marrow and lymphatic tissue. The clinical course is variable with early stage disease having life expectancy greater than 10 years, but patients with more advanced disease have a median survival of 18 months to 3 years. The current standard therapy of fludarabine, cyclophosphamide and rituximab (FCR therapy) has improved complete responses and overall survival (1), but relapse is inevitable and the therapy is associated with significant toxicities. Therefore, identifying new agents with novel mechanisms of action that complement current chemotherapies and abrogate the CLL drug-resistance factors will be necessary if further improvements in CLL patient outcomes are to be realized.

The tumour microenvironment is essential for tumour proliferation, survival, angiogenesis, and metastasis in a range of malignancies including CLL (2). Studies using heavy water suggest that CLL consists of a pool of quiescent cells circulating in the peripheral blood together with a reservoir of proliferating cells within the bone marrow and lymph nodes (3, 4). This suggests a pro-proliferative environment is located within bone marrow and lymph node whereas CLL cells within blood are associated with long term survival. CLL proliferation, survival, homing and drug resistance (5-12) have been attributed to numerous factors such as chemokines, cytokines and direct cell-cell interactions mediated via adhesion molecules in the lymph node, spleen and bone marrow microenvironment which suggests these factors may represent important therapeutic targets. For example, the Bruton tyrosine kinase (BTK) inhibitor, ibrutinib, impairs B-cell receptor- and chemokine-controlled
retention of CLL cells in the marrow and lymph nodes. This deprives the CLL cells of their microenvironmental survival signals leading to disease regression (13). Consequently, there is growing interest in targeting the survival cues associated with the tumor-microenvironment. Understanding the molecular and biological basis for these microenvironment-mediated responses will be critical to understanding CLL and developing more potent or effective therapies (12).

Our previous work has concentrated on the development of a long term culture system where culturing CLL PBMCs at high density provides a significant survival advantage to CLL cells (14). This culture system was also confirmed by the recent report of Seiffert et al (15). Having established that culturing CLL PBMCs at high density gave a significant survival advantage to CLL cells we set about investigating what cell-cell interactions might be important for survival by assessing expression levels of cell surface markers. Here we report a significant upregulation of several markers CD184 (CXCR4), CD26, CD40, CD58, CD103, and CD62L (L-selectin) on the surviving CD5+/CD19+ CLL cells. On the basis of CD62L’s known role in normal lymphocyte migration (16, 17), and initial reports of its prognostic significance in CLL (18), we hypothesised that CD62L may be important in microenvironmental retention, homing or survival of CLL cells and may offer a potential therapeutic target. We i) examined the expression of CD62L in CLL, ii) investigated the effect of anti-CD62L antibody therapy on CLL cell survival in vitro, and iii) assessed the cytotoxicity of CD62L blockade in combination with the standard agents used in the treatment of CLL. Results presented in this study support the potential of anti-CD62L as a therapeutic agent.
Methods and Materials

Patients and Cell Purification. Blood was collected into EDTA tubes from healthy donors or patients with CLL after informed consent according to protocols approved by the Princess Alexandra Hospital (PAH) Human Research Ethics Committee. Diagnosis of CLL was made according to NCI criteria. CLL patients were either untreated (39 samples) or a median of 24 months after their last treatment (range 12-57 months, 14 samples; Supplementary Table 1). Mononuclear cells were isolated by density-gradient centrifugation over Histopaque-1077 (Sigma-Aldrich, St Louis, MO).

CLL culture and cell viability. Purified PBMCs were resuspended in RPMI 1640 medium (Gibco Invitrogen, 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 20-60x10⁶/ml. It should be noted that these long term cultures of PBMCs contain non-CLL cells derived from circulating white blood cells plus an adherent population of cells which includes nurse-like cells and endothelial cells. Cell viability was performed by trypan blue exclusion and survival was expressed as number of viable cell counts relative to initial plating counts or normalised to untreated CLL PBMC survival. Cell viability and apoptosis was determined by flow cytometry using Annexin V/PI staining kit (Roche, Australia) according to the manufacturers’ instructions. CD19⁺ CLL cells were purified using MACS® cell separation and CD19 Microbeads (Miltenyi Biotec, Germany) according to the manufacturer’s protocol.

Flow Cytometry screening analysis of surface antigens. CLL PBMCs from three patients were screened for the expression of 71 cell surface antigens (outlined in Supplementary Table
2) directly after isolation from the patient and after three weeks of high density culture 
(2x10^7/ml). Cells were washed with phosphate buffered saline (PBS) and analysed on a BD 
LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ). Viable cells were gated based 
on forward and side scatter profile. Data were analysed using BD FACSDiVa software (BD 
Biosciences). Following initial FACS screen, CD62L expression was confirmed on additional 
patient samples using flow cytometry. Cells were incubated with APC-conjugated anti-CD19, 
FITC-conjugated anti-CD5 (both from BD Biosciences) and PE-conjugated anti-CD62L 
antibodies (Biolegend, San Diego, CA) and analysed on a FACSCanto (BD Biosciences). 
Data were analysed using BD FACSDiVa software.

In vitro CD62L Assays. The effect of neutralising CD62L was examined by culturing CLL 
PBMCs or PBMCs isolated from healthy donors in the presence of LEAF™ Purified anti-
human CD62L antibody (DREG56; 0.1µg/ml; Biolegend), anti-human CD62L antibody 
(DREG200, 0.1µg/ml, ebioscience, San Diego, CA) or LEAF™ Purified Mouse IgG1, κ 
Isotype Control Antibody (0.1µg/ml; Biolegend) and cell viability was examined as described 
above. For co-culture experiments, HUVEC or HS-5 cells were seeded at a density of 2 000 
cells/well in 96 well plates 24 hours prior to addition of CLL PBMCs with/without CD62L 
antibody.

Chemotherapy combination assays. To examine the therapeutic potential of CD62L 
antibody therapy, CLL PBMCs were cultured either alone, or in the presence of HS-5 cells, 
with varying combinations of the following agents; CD62L antibody (DREG56; 0.1µg/ml), 
Rituximab (10µg/ml, Mabthera®, Roche, Italy) both on day 0 and fludarabine (1µg/ml, 
Fludara®, Schering, Italy) and/or mafosfamide (1µg/ml; Santa Cruz, Dallas, TX) on day 4.
Western Blotting. Cultured CLL cells were lysed in RIPA buffer containing phosphatase and protease inhibitors and electrophoresed onto 10% SDS-polyacrylamide gels. Membranes were developed using SuperSignal West Pico Chemiluminescence substrate (Thermo Scientific, Rockford, IL). The following antibodies were used: PARP, LC3 A/B, pAKT (Ser 437) XP, total AKT, phosphor-p44/42 MAPK (Thr 202/Tyr 204), total MAPK (all from Cell Signalling, Danvers, MA), GAPDH (Sigma-Aldrich, Germany) and B-actin (Sigma-Aldrich).

Immunofluorescence Staining and Confocal Microscopy
Ethics Committee approval was obtained to use the redundant portion of bone marrow trephine biopsies from untreated CLL patients. Sections from an uninvolved lymphoma bone marrow, from a normal lymph node and from an uninvolved node from a lymphoma patient were used as controls. Primary antibodies include mouse monoclonal anti-human-CD62L (Clone 9H6; Novocastra, Germany), mouse monoclonal anti-human-CD20 (Clone L26; Dako, Denmark). Secondary antibodies include Alexa Fluor® 555 goat-anti-mouse and Alexa Fluor® 488 goat-anti-mouse antibodies (Invitrogen). Sections were counterstained with DAPI for nuclear staining before being mounted using Vectashield® (Vector Laboratories, Inc., Burlingame, CA). Immunofluorescent signals were viewed through a Carl Zeiss LSM 510 Meta confocal microscopy system (Carl Zeiss, North Ryde, Australia) and analysed using Zen imaging software.

Data Analysis and Statistics. Results are shown as mean ± SEM of at least 3 experiments each performed in triplicate. For statistical comparison, the Student unpaired or paired t test was used, two tailed with 95% confidence intervals. Pearson’s correlation tests were used for examining the relationship of clinical parameters with response to CD62L treatment.
Response to CD62L antibody treatment was normalised to viability in untreated cultures prior to correlation analysis. Findings were considered significant if $p \leq 0.05$. 
Results.

CD62L expression is upregulated on CLL cells in long-term culture: Immuno-phenotyping was carried out on three CLL patients using 71 antibodies, comparing the percentage positive cells at day 0 with those after three weeks in culture. This analysis was performed on suspension cells only and did not include adherent cell populations and nurse like cells. CD5⁺/CD19⁺ cells constituted over 80% of the total cells in culture at day 0 and this was maintained at week 3, indicating persistence of the CLL population (Figure 1A and 1B). Significant changes were observed for 25 surface antigens, with increases observed in the expression of CD26, CD40, CD58, CD62L and CD103 (Figure 1A) and decreased expression found in the other markers including CD11c, CD32, CD49f, CD62P, CD80, CD106, CD140a, CD141, CD184, CD206 and CD273 (Figure 1B). The complete data set is presented in Supplementary Figure 1. Some of the observed differences in cell surface markers may be due to changes in the non-CLL cell population during culture. For example, CD206 is expressed on immature dendritic cells and macrophages, cells that are expected to be lost in culture. However, the main power of our screening approach is that we were able to identify those molecules for which expression is highly upregulated during the culture of CLL cells such as CD62L (Fig. 1A). The veracity of the screen was also validated by the identification of molecules previously identified in CLL biology including CD58 (LFA3), a cell adhesion molecule whose increased expression has been associated with advanced disease state (19) and CD184 (CXCR4), the receptor for SDF-1 which has been shown to protect cells from apoptosis in vitro (20). Several of the other molecules which exhibit significant changes in expression are related to cell adhesion including a downregulation of CD18 (integrin β-2) which is the beta subunit of 4 structures, of which 2 are also
differentially down regulated in our screen, CD11a and CD11c. Overall, the most significantly upregulated marker was CD62L.

In order to directly confirm that the increased levels of CD62L were expressed on CLL cells we undertook double-labelling of CLL PBMCs with CD19 and CD62L antibodies and monitored their levels over 7 days in culture. It was found that the number of CD19⁺/CD62L⁺ cells was below 1% when isolated from the patient, but rapidly climbed, with an increase to 36.8% within 24 hours and this level continued to increase to above 80% by day 7. A representative result is shown in Figure 2A. Examination of nine further primary cultures showed that there was a significant increase in CD62L levels in each case, with the average CD19⁺/CD62L⁺ population moving from 12±6% to 81±5% (p<0.0001; Figure 2B).

As CD62L has previously been shown to be expressed on a range of other lymphocytes including T cells (21) we undertook flow cytometry analysis of both B (CD5⁺/CD19⁺) and T cells (CD5⁺/CD19⁻) within the same PBMC culture to determine which cells were upregulating the CD62L. Our data show that it was in fact the CLL cells that became positive for CD62L over time rather than T cells within the culture (Figure 2C). The upregulation of CD62L was maximal by day 5 and was maintained in CD5⁺/CD19⁺ cells for up to 21 days (Figure 2C). It was also critical to demonstrate that the specificity of this increased expression was an inherent property of CLL cells and not a characteristic of normal B cells. Therefore, PBMCs from a healthy 60 year old female volunteer were cultured for 7 days at the same seeding density as CLL PBMCs and the level of CD62L expression measured by flow cytometry. The level of CD19⁺/CD62L⁺ cells increased minimally from 2.8% to 5.8% over 7 days which indicates increased CD62L expression is a feature of CLL cells rather than normal B cells (Figure 2D). The induction of CD62L was also examined in CD19 purified CLL cells to determine if accessory cells within the PBMC culture were essential for the increased expression of CD62L. This is shown in Figure 2E where there is
no significant difference in CD62L expression between CLL cells in PBMC culture and CD19 purified cells from the same patients. The expression of CD62L was also examined on viable cells using Annexin V staining after 7 days in culture to confirm that this upregulation was related to CLL cell survival. Only cells which were low in Annexin V positivity showed expression of CD62L (Figure 2E). Finally, the induction of CD62L expression was not due to the expansion/proliferation of the CLL fraction since BrdU labelling studies indicated that the proliferation index could not account for the large expansion in CD62L positive cells. Thus, the increased expression of CD62L is due to a time-dependent induction of expression of CD62L on CD5+/CD19+ CLL cells.

**CD62L expression is upregulated in CLL cells in lymph node and bone marrow:** As CD62L is involved in homing and migration of lymphocytes to lymphoid organs, we examined the expression of CD62L in bone marrow and lymph node biopsies from CLL patients using immunofluorescent staining. We detected CD62L expression on a large proportion of CD20 positive B-cells in lymph node sections from CLL patients (Figure 3A). Sections from a normal lymph node and from a non-involved lymph node from a lymphoma patient served as controls, with a very small number of CD20 positive cells positive for CD62L in the normal lymph node control and no CD62L expression detected in the lymphoma control. The CD62L expression in the bone marrow was even more striking with a high proportion of CD20 positive cells co-expressing CD62L (Figure 3B). Conversely, a few CD20 positive cells were present in the lymphoma control bone marrow and there was no CD62L expression evident. To examine if there was any association with expression of CD62L in the lymph node and bone marrow compartments compared with the periphery, CD62L expression was examined on CLL PBMCs from matched patients used in above immunofluorescence studies (Figure 3C). CD62L expression in CLL PBMCs varied from
0.9% to 43.5% between the 3 patients used, but this positivity did not directly reflect the CD62L expression observed in the lymph node and bone marrow. For example, CLL patient 16 had high expression of CD62L in the bone marrow but very low CD62L expression in CLL PBMCs (0.9%). This was also seen for CLL patient 17, with high expression of CD62L on CD20 positive cells in the bone marrow, but only 17.3% CD62L positivity in CLL PBMCs. These results indicate the presence of CD62L in proliferation and survival niches involved in CLL.

**Blocking CD62L promotes apoptosis of CLL cells:** Given the highly induced expression of CD62L in surviving CLL cells we examined the effect of a neutralising CD62L antibody. Dose response analysis (0-2μg/ml) was initially performed to determine the optimal dose of anti-CD62L antibody that induced cell death. Maximal response was achieved at 0.1μg/ml (data not shown). This trend was observed regardless of patient white cell counts. Initially, 7 CLL patients’ PBMCs were cultured as described previously in media containing either anti-CD62L (DREG-56 clone) or an isotype control antibody, with PBMCs from age matched healthy individuals acting as controls. The neutralisation of CD62L resulted in a significant loss of CLL cell survival (11.27%±1.638) compared to untreated CLL PBMCs (49.12±3.554; p<0.0001; Figure 4A). CLL cells treated with an isotype control antibody had the same survival profile as untreated CLL PBMCs with 42.87% and 49.12% survival respectively (p=0.1649). Importantly, PBMCs from normal healthy controls were unaffected by treatment with anti-CD62L antibody reinforcing that the response is restricted to CLL cells. Further testing of 36 CLL patient samples confirmed the role of CD62L blockade in CLL cell survival with all patients showing a reduction in surviving cells (Figure 4B) with a mean reduction of 69% (p=0.001). To determine the mechanism underlying the reduced cell survival in response to CD62L blocking we measured apoptosis using flow cytometry. It was
observed that cells were undergoing apoptosis as a result of the neutralisation of CD62L (Figure 4C) with an increase in late apoptotic cells from 5.2% to 71.1% when treated with the CD62L antibody compared to untreated PBMCs and similarly when compared to the isotype control. Significantly, the CD62L antibody treatment was causing a specific reduction of CD5+/CD19+ cells with a 49% reduction in the proportion of CLL cells in the PBMC culture when compared to untreated PBMCs (Figure 4D). The specificity of targeting CD62L was confirmed by using a different neutralising antibody, DREG-200, which, like DREG-56 targets the lectin binding domain of CD62L. Utilizing the same culture and antibody treatment conditions, DREG-200 significantly reduced the survival of CLL cells by 55.93%, although not as dramatically as observed for DREG-56 with a 45.33% reduction (p<0.01 for both DREG56 and DREG200; Figure 4E). In addition, annexin V/PI staining confirms that both antibodies initiate increased apoptosis of CLL cells and corroborates the cell survival data (Figure 4F). Together these data indicate that treatment with a CD62L antibody results in apoptosis in a CLL cell specific manner.

To further dissect the mechanism of CD62L antibody mediated cytotoxicity, we examined the induction of common survival pathway markers such as PARP cleavage, MAPK (total and phosphorylated) and, Akt (total and phosphorylated). However, there was no difference observed in expression levels, or activation state between untreated CLL PBMCs and CD62L antibody treated cells (Figure 5A, 5C). In addition, we observed no changes for the autophagy marker, light chain 3 (LC3 A/B) expression, (Figure 5B) following treatment. These data indicate that the apoptosis-inducing actions of anti CD62L are independent of objective changes in the activity of the AKT, MAPK, PARP or autophagy pathways.
Association of response to CD62L blockade and clinical parameters: Having established the specific effect of CD62L antibody treatment on CLL PBMC cultures, we examined if any associations were apparent between anti-CD62L induced CLL death and clinical parameters. The most significant association was a positive correlation between CD62L antibody response and CD5+/CD19+ percentage (p=0.0142) and lymphocyte count (p=0.0533) at time of sample collection and a negative correlation for haemoglobin (p=0.0348; Supplementary Figure 2A-C). Together these associations indicate a relationship between low leukemic burden and more effective *in vitro* antibody response. However, CLL PBMCs isolated from patients with unmutated immunoglobulin heavy-chain variable-region (IgVH) gene mutations had a superior response to the CD62L antibody compared to those who were mutated (Supplementary Figure 2F). Additionally, no significant associations were observed between CD62L response and CD38 expression (Supplementary Figure 2D), Zap70 expression (Supplementary Figure 2E) or Rai stage (Supplementary Figure 2G). Other clinical parameters which showed no correlation with effectiveness of CD62L antibody treatment include lymphocyte doubling time, time till first treatment, age, and platelet count, albumin, hypogammaglobulinaemia, LDH and beta-2 microglobulin levels (Supplementary Figure 3).

Effect of CD62L blockade is not abrogated by co-culture with supporting microenvironmental cells: CLL cell survival has previously been shown to be enhanced by co-culture of CLL cells with both bone marrow stromal cells (HS-5) and human umbilical vein endothelial cells (HUVEC) by recapitulating the pro-survival signals that may exist in the leukemic microenvironment *in vivo* (22, 23). As CD62L is overexpressed in surviving CLL cells *in vitro* and CD62L-specific antibodies are able to induce apoptosis we examined the possibility that the cytotoxic response to CD62L antibody could be antagonised by a pro-survival interaction of CD62L expressed on CLL cells with stromal cells. HS-5 cells provide...
a survival advantage over CLL PBMC culture alone with 9.5% increase in overall cell survival, but do not protect against anti-CD62L antibody treatment with 37.6% and 38.9% reduction in survival for CLL PBMCs culture and HS5-5 co-culture respectively (p<0.0001; Figure 6A). Similar results were observed for HUVEC co-culture experiments (Figure 6A). These results indicate that anti-CD62L therapy may be effective in vivo where pro-survival micro-environmental interactions may contribute to the oncogenic expansion of CLL cells.

**Therapeutic potential of CD62L antibody therapy:** Since CD62L appears to modulate the survival response of CLL cells in vitro, we extended this to determine whether CD62L blockade may also modulate sensitivity to conventional chemotherapeutics used in the treatment of CLL. Experiments were performed combining maximal doses of CD62L antibody (0.1µg/ml) added on day 0 and maximal cytotoxic doses of fludarabine (1µg/ml) added on day 4 post CD62L antibody treatment. Cell survival was then examined at 7 days. These experiments showed that fludarabine and CD62L antibody, were similar in their cytotoxic potency with a 55.5% and 43.65% reduction in survival, respectively (Fig. 6B). However, a combination of CD62L antibody plus fludarabine gave significantly greater cytotoxic responses compared with each drug given alone with a 70.53% reduction in cell survival (Fig. 6B). This response was not altered when fludarabine was added prior to the addition of the CD62L antibody (Data not shown). This trend was also observed when CLL PBMCs were cultured with HS-5 cells (Figure 6B). Similarly, when CD62L antibody responses were compared to mafosfamide (1µg/ml), a cyclophosphamide analog used in in vitro experiments, similar potencies were observed when used as single agents, but an additive effect when used in combination (Figure 6C). These responses were maintained when cultured with HS-5 cells. However, the cytotoxic responses with CD62L antibody treatment and rituximab (10µg/ml) showed no significant difference when used either as
single agents or in combinations (Figure 6D). However, when cultured with HS-5 cells, there
was a significant difference between rituximab treatment alone and the combination with
CD62L antibody (Figure 6D). Given the additive effect observed for CD62L with fludarabine
or mafosfamide, we examined the consequence of combining the three agents. There was a
significant increase in cytotoxic responses when CD62L was combined with both fludarabine
and mafosfamide compared to either each agent alone, or any two agents combined (Figure
6E). Next, we examined if the addition of CD62L antibody treatment was comparable to
current chemoimmunotherapy strategies that use a combination of fludarabine,
cyclophosphamide and rituximab therapy (FCR). There was no significant increase in the
response between fludarabine, mafosfamide and rituximab combination and fludarabine,
mafosfamide and CD62L antibody combinations with survival reduction of 74.4% and
81.36% respectively (p=0.140, Figure 6F). There was no significant advantage of combining
all four agents (85.56% reduction in cell survival). Flow cytometric analysis of the surviving
CLL cells following treatment with CD62L antibody, fludarabine, mafosfamide or rituximab
demonstrates that CD62L blockade is the most efficient in specifically targeting CLL cells
with a reduction from 80.2% to 62.7% after 7 days (Figure 6G). Conversely, fludarabine
(85.2%), mafosfamide (88.0%) and rituximab (86.5%) showed no specific reduction in CLL
percentages. These data indicate that combining the cytotoxic activity of current CLL
therapies with the anti-survival activity of CD62L inhibition may enhance cytotoxic
responses of CLL cells.
Discussion

CLL is a B cell malignancy characterised by aberrant control of apoptosis. Recently, we and others have demonstrated that death, in vitro, could be reduced by culture of CLL cells at high density and/or in the presence of accessory and nurse like cells (14, 15). These data and other accumulating evidence indicate that CLL cell resistance to apoptosis is attributable to micro-environmental factors mediated via cell:cell interactions and/or dysregulation of pro-apoptotic or pro-survival cytokines, and their cognate receptors and signalling pathways. In this study we show, for the first time that i) survival of CLL cells, in vitro, is accompanied by profound increases in the expression of CD62L, ii) pro-survival signals from stromal cells are CD62L-dependant as inhibition of CD62L, by therapeutic antibodies, reduces CLL cell survival, iii) that CD62L-induced survival pathways are independent of those pathways mediating fludarabine or mafosfamide-induced cytotoxicity as CD62L antibody treatment potentiates the cytotoxic effects of fludarabine, mafosfamide beyond those observed using a maximal dose of fludarabine or mafosfamide alone and, iv) CD62L antibody treatment, in vitro, can overcome the pro-survival signals provided by stromal cells. This latter point is of particular relevance since we show that CD62L expression is profoundly overexpressed on malignant B-cells located within lymph nodes and the bone marrow particularly in the proliferation centres (pseudofollicles). The malignant B-cells residing in the bone marrow and lymph node compartments are particularly resistant to apoptosis due to the pro-survival signals within these niches. Thus, CD62L is a novel prosurvival effector that may represent an attractive therapeutic target in CLL.

CD62L (L-selectin, LAM-1, LECAM1, SELL) is a member of the selectin family of adhesion molecules and is known to play an important role in the trafficking/homing of
lymphocytes to the lymph node. For example, CD62L binds several ligands involved in homing and migration such as, CD34, GlyCAM-1, and MadCAM-1 (21). Moreover, high CD62L and CXCR4 expression on lymphocytes is needed to migrate to lymph nodes and bone marrow (24). Once in the lymph nodes and bone marrow environment, lymphocytes migrate into the proliferating centres (PCs) under the influence of chemokines such as CXCL12 (25). Conversely, lower levels of CD62L expression on CLL cells has been associated with an impaired capacity to migrate under endothelial cells in vitro (26). Furthermore, lower CD62L and CXCR4 expression is necessary for CLL cells to exit these microenvironmental niches (27). Consistent with these observations on the role of CD62L in lymphocyte homing it has also been shown that BCR activation results in downregulation of CD62L and CXCR4 (27). Thus, the dysregulation of CD62L expression on CLL cells is likely to contribute to the pathological disturbance in total CLL numbers and the accumulation of CLL cells in the peripheral blood and lymph nodes.

The results of this study demonstrate that in vitro addition of anti-CD62L to primary CLL results in apoptotic loss of CD5+/CD19+ cell comparable to fludarabine-induced cytotoxicity. More importantly PBMCs from normal healthy controls were unaffected by treatment with anti-CD62L antibody reinforcing that the response is restricted to CLL cells. This effect of CD62L blockade is not abrogated by the presence of stromal cell line HS-5 suggesting that anti-CD62L therapy may be effective in vivo where pro-survival micro-environmental interactions may contribute to the oncogenic expansion of CLL cells. There was a significant increase in cytotoxic responses when CD62L was combined with both fludarabine and mafosfamide compared to either each agent alone, or any two agents combined. These findings indicate that different pathways in survival/death pathways in response to CD62L antibody or fludarabine and mafosfamide exist. These data also highlight
the potential therapeutic value of combining anti-CD62L therapies with current chemotherapy agents in CLL patients.

The mechanism by which CD62L antibody produces cytotoxicity to CLL cells is currently unknown. Since we have previously shown that CLL cell survival, in vitro, is modulated by secreted factor(s) as well as cell:cell interactions, it is likely that the mechanisms involved are complex and may involve multiple independent signaling events or independent signaling events that converge on a common pro-survival pathway. Significantly, we found no evidence for constitutive activation or induction of common survival pathways such as AKT or MAPK signaling pathways in CLL cells taken from patients or following culture in vitro. Furthermore, addition of a neutralizing CD62L antibody failed to alter AKT or MAPK activity. These data indicate that CD62L-mediated survival is independent of alterations in AKT and MAPK signaling. Given that cytotoxicity induced by CD62L neutralizing antibody took several days to occur, we considered the possibility that it may be driven by autophagy. However, death induced by CD62L antibody was not associated with changes in the expression of the known autophagy marker, LC3A/B. Thus, CD62L signaling was independent of AKT and MAPK pathways and was linked to the suppression of apoptosis. Our data suggest that there may be other hitherto unknown roles for CD62L in CLL.

Finally, the present study identified a number of cell surface markers associated with primary CLL cell survival. Whilst, these markers were not examined in detail, their expression served to validate our approach since many of these cell surface markers have previously been reported to play roles in CLL cell survival. For example, we identified increased levels of expression of CD184 (CXCR4), CD26, CD40, CD58, CD62L, and
CD103, CD60L and CXCR4 are key molecules in CLL cell trafficking. We observed that CD40 was increased in surviving CLL cells. Interaction between B cells and T_h cells in lymph node proliferating centres is mediated via CD40 cross-linking with CD40L to activate protein kinases such as Lyn and Syk and such interactions have been observed in CLL patient lymph nodes (28). The fact that CD26 was increased in surviving CLL cells is indicative of B cell activation (29, 30) which is consistent with the up-regulation of CD58, another marker of co-stimulation. It has been shown that incubation of CLL cells with human CD40L-expressing T cells results in increased CD54, CD58, CD80 and CD86 (31). Further study of these molecules may provide insight into the pathways that drive the dysregulation of B-cell survival/apoptosis in CLL.

In conclusion, our work establishes an important role of CD62L in different prosurvival stimuli provided to CLL cells by the microenvironment. Our data contribute to the establishment of CD62L inhibition as a rational therapeutic principle in CLL. Anti-CD62L antibody has marked in vitro activity against human CLL cells at concentrations that are readily attainable in the clinic. In addition to the cytotoxicity of CD62L in CLL, our study adds the important prospect of preventing survival of drug-resistant CLL cells in protective niches by direct disruption of stromal signals by inhibition of CD62L.
Figure Legends.

Figure 1. Cell surface markers showing differential expression. CLL PBMCs were cultured at high density for three weeks and expression of 71 cell surface molecules was examined and compared to baseline levels using flow cytometry. Paired t tests determined which cell surface markers were differentially expressed, either an increase (A) or a decrease in expression observed over time in culture (B). Data is displayed as the mean ± SEM (n=3; *p < 0.05 **p < 0.01 ***p < 0.001).

Figure 2. Increasing CD62L expression on CLL cells in vitro. (A). CLL PBMCs were cultured at high density and expression of CD62L on CD19 positive cells was examined using flow cytometry over 7 days in culture. Several patient samples were used and this data is representative of one patient. (B). An additional nine patients were examined for expression of CD62L on CD19 positive cells after 7 days in culture. (C). Flow cytometry analysis of CD62L expression on CD19+/CD5+ CLL cells and CD5+ T cells throughout 14 days in culture. Data is representative of one patient. (D). PBMCs from healthy donors were examined for expression of CD62L before and after 7 days in culture using flow cytometry. A total of 3 donors were examined with 1 donor represented. (E) Induction of CD62L expression was also examined on CD19 purified CLL PBMCs pre and post in vitro culture. Data is displayed as the mean ± SEM. (F) Flow cytometry analysis of CD62L expression on annexin V negative cells. Data is representative of one patient.

Figure 3. CD62L is detected in CD20+ cells in CLL lymph nodes and bone marrow trephines. Representative sections were presented for 2 CLL lymph nodes, an uninvolved lymphoma control, and a normal lymph node control trephine (A). Representative sections were presented for a CLL trephine and an uninvolved lymphoma control (B). Confocal
microscopy was performed for double-staining of CD62L (green) and CD20 (red). Images were presented for sections incubated with the positive antibodies (CD62L and CD20) and with the isotype control antibodies (the neg Ab control). (C) Flow cytometry analysis of PBMCs from time-matched patients used in (A) and (B) were examined for CD19 and CD5 and CD62L positivity.

**Figure 4. Blocking CD62L results in a specific reduction of CLL cells.** (A). CLL PBMCs or PBMCs isolated from healthy donors were cultured in the presence of anti-human CD62L antibody (DREG56) or an isotype control antibody, both at a concentration of 0.1µg/ml, and cell survival examined after 7 days. Data is displayed as the mean ± SEM and is representative of 7 CLL patients and 4 healthy donors, each performed in triplicate; ***p < 0.001. (B). As for (A). but examined in an additional 37 patients. Data is displayed as the mean ± SEM; a paired t test was performed with p ≤ 0.0001. (C). Apoptosis was examined by Annexin V/PI staining on cells cultured as described above and examined using flow cytometry. Percentages represent late apoptotic cells; data is representative of 1 patient. (D). Flow cytometry analysis of CLL PBMC cultures treated with CD62L antibody were examined for CD19 and CD5 positivity after 7 days in culture. The percentage is representative of CLL cells and data is representative of one patient. (E). Antibody response was validated using a different CD62L antibody clone (DREG200, 0.1µg/ml) and cell survival examined after 7 days. Data is displayed as the mean ± SEM and is representative of 3 CLL patients, each performed in triplicate; **p < 0.01. (F). Annexin V/PI staining was performed on CLL PBMCs cultured as in (E) with percentage representing late apoptotic cells. Data is representative of 1 patient.

**Figure 5. CD62L related cytotoxicity is not dependent on Akt, MAPK or autophagy pathways.** CLL PBMCs were cultured with or without CD62L antibody and protein
expression of PARP (A), LC3A/B (B) and total and phosphorylated Akt and MAPK (C) examined by immunolot. Representative blots from 1 patient are shown. Analysis was performed for a total of 3 patients.

Figure 6. Therapeutic potential of CD62L antibody therapy. (A). CLL PBMCs were cultured for 7 days either alone or co-cultured with HS-5 or HUVEC cells for 1 week with anti-human CD62L antibody. Overall cell survival was examined by trypan blue exclusion and data is displayed as the mean ± SEM (n=7 for HS5 and n=3 for HUVEC; *p < 0.05 ***p < 0.001). CLL PBMCs were cultured alone or with HS5 cells and treated with CD62L antibody on day 0 either alone or in combination with fludarabine (B) mafosfamide (C) or rituximab (D) and cell survival examined on day 7. Data is displayed as mean ± SEM (n=8; **p < 0.01 ***p < 0.001) (E). CLL PBMCs were cultured with either CD62L antibody, fludarabine or mafosfamide alone or in combinations as shown and survival examined. Data is displayed as mean ± SEM (n=8; **p < 0.01 ***p < 0.001) . (F) CLL PBMCs were cultured using the therapy combinations as shown and cell survival examined after 7 days. (G). CD19 and CD5 positive cells were examined after 7 days following treatment using flow cytometry.
References


23. Kay NE, Shanafelt TD, Streege AK, Lee YK, Bone ND, Raza A. Bone biopsy derived marrow stromal elements rescue chronic lymphocytic leukemia B-cells from spontaneous and drug induced cell death and facilitates an "angiogenic switch". Leuk Res. 2007;31(7):899-906.


Figure 1.

A

B

% Positive Cells

Day 0

Week 3

CD3, CD19, CD4, CD8, CD25, CD69, CD44, CD141, CD162, CD103
Figure 2.

A. Day 0
   Day 1
   Day 3
   Day 7

B. % CD62L Positive Cells
   Before Culture
   After Culture
   CLL Patients

C. % CD62L Positive Cells
   CD19+/CD5+ CLL Cells
   CD5+ T Cells
   Days in Culture
   n = 1

D. Day 0
   Day 7

E. % Positive Cells
   CLL PBMC
   CD19 purified CLL PBMCs
   Day 0
   Day 5
Figure 4: Displaying various experimental results.

A. Graph showing % Staining of CLL cells in different groups.
B. Scatter plot comparing % Staining of CLL cells with CD38 Ab.
C. Flow cytometry images showing Annexin V expression.
D. Flow cytometry images displaying CD5 levels.
E. Graph illustrating % CLL cells in different conditions.
F. Flow cytometry images with DREG56 Ab.
Figure 5.

A. 

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B. 

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C. 

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CD62L as a therapeutic target in chronic lymphocytic leukemia
Melinda Burgess, Devinder S. Gill, Richa Singhania, et al.
Clin Cancer Res Published OnlineFirst August 15, 2013.

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