Regulation of CD38 in proliferating chronic lymphocytic leukemia cells stimulated with CD154 and interleukin-4

Shaun Willimott, Maria Baou, Sarah Huf, Silvia Deaglio, Simon D. Wagner

From the Division of Investigative Sciences, Department of Haematology, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 ONN, UK (SW, MB, SH, SDW); Laboratory of Immunogenetics, Department of Genetics, Biology and Biochemistry, University of Torino, Italy (SD).

Funding: this work was supported in part by grants from the Leukaemia Research Fund and Lymphoma Research Trust to SDW.

Manuscript received February 5, 2007.
Manuscript accepted July 23, 2007.

Correspondence:
Simon D. Wagner, Department of Haematology, Imperial College London, Hammersmith Hospital, Du Cane Road, London, W12 ONN, UK.
E-mail: simon.wagner@imperial.ac.uk

ABSTRACT

Background and Objectives

Chronic lymphocytic leukemia (CLL) cells, like normal B-cells, exist in two populations *in vivo*: quiescent cells in the peripheral circulation and proliferating cells in lymph nodes. The surface marker CD38 has roles in cell adhesion and signaling. Its expression correlates with poor clinical outcome and is associated with expression of the signaling intermediate ZAP-70, which is also a marker of poor prognosis. We investigated the regulation of CD38 and ZAP-70 in proliferating CLL cells.

Design and Methods

We cultured CLL cells on a stromal cell layer that maintains viability and also with some stromal cells expressing CD40 ligand (CD154) in order to measure changes in expression of CD38 and ZAP-70.

Results

We demonstrated up-regulation of CD38 expression by CD154. The degree of up-regulation did not correlate with clinical stage or mutational status. In addition in the majority of cases tested ZAP-70 expression increased in parallel with up-regulation of CD38 although discordant cases were also observed.

Interpretation and Conclusions

Overall we demonstrated that regulation of CD38 in CLL is dynamic and dependent on signals from CD154 and a stromal cell layer. We speculate that CD38 and ZAP-70 are expressed in lymph node leukemic cells in both good and poor prognosis patients, but, in cases with good clinical outcome, these molecules are down-regulated in the peripheral blood whereas in cases with poor prognosis their expression is maintained.

Key words: CLL, CD40 ligand, CD38.

Haematologica 2007; 92:1359-1366. DOI: 10.3324/haematol.11340

Chronic lymphocytic leukemia (CLL) is the commonest B-cell lymphoproliferative disease in the western hemisphere. There is a high degree of variation in the clinical course with some patients remaining asymptomatic for many years whilst others rapidly require treatment. A number of molecular markers including cytogenetics abnormalities,[1] immunoglobulin gene mutational status,[2,3] ZAP-70 expression[4,5] and CD38 expression[6-8] allow the prognosis of asymptomatic patients to be predicted. The regulation of expression and functions of these prognostic markers must be related, either directly or indirectly, to the clinical evolution of CLL and this relation is currently being intensively investigated. One hypothesis is that proliferation of CLL cells in patient with a poor prognosis is greater than that of cells from cases with a good prognosis and this is supported by data showing that signaling through the B-cell receptor of unmutated-CLL is greater than that of mutated-CLL.[9] Similarly, transduction of ZAP-70 in CLL cells increased proliferation.[10,11] Signaling through CD38 has also been shown to drive CLL cell proliferation[12,13] suggesting that signals delivered through this surface molecule cause cell division in poor prognosis CD38-expressing cases but not in CD38-negative cases.

The ligand of CD38 is platelet endothelial cell adhesion molecule–1 (PECAM-1 or CD31),[14,15] which is expressed by both CLL cells[16,17] and endothelial tissues. As well as driving proliferation, CD38 may, therefore, also have a role in cell adhesion. CD38 carries out an ADP-ribosyl cyclase reaction, catalyzing the cyclization of NAD to produce cyclic ADP-ribose, but the significance of this for the biology of CLL is not known. Flow cytometry shows that the expression of CD38 on CLL cells in lymph nodes is greater than that on peripheral blood cells.[18] CLL lymph nodes contain proliferation centers, containing T cells and dendritic cells as well as proliferating CLL cells and this suggests that CD38 expression is regulated by the lymph node microenvironment.

The microenvironment is critical for the growth of CLL cells,[19,20] and several systems, using different stromal cell layers, have been used[21-23] *in vitro* to mimic these conditions. Seminal works[24,25] demonstrated that cross-linking CD40 in the context of a stromal layer (human fibroblast cell line) produced proliferation of normal B cells, which could be enhanced by the addition of interleukin-4 (IL-4). In a small *in vitro* study CLL cells responded by proliferation to the specific combination of IL-4 with CD40 stimulation.[26] The combination of IL-4 with B-cell receptor signaling did not produce this effect. In order to study both quiescent (representing peripheral blood leukemic cells) and proliferating cells we have characterized a dual cell culture system[27] that utilizes non-transfected mouse fibroblast L-cells (NT-L) to maintain viable CLL cells and L-cells transfected with CD40 ligand (CD154) combined with IL-4 (CD154/IL-4 system) to drive their proliferation.

Design and Methods

Patients

Patients with both early stage and advanced disease were studied (*Supplementary Table 1*), provided that they had a white cell count >50×10^9/L. At the time of study no patient had been treated for 3 months. Blood was also obtained from healthy donors. Local research ethics committee approval was obtained. Immunoglobulin gene mutational status was determined by polymerase chain reaction (PCR) amplification of the rearranged immunoglobulin gene and sequencing;[3] ≥98% homology indicates unmutated immunoglobulin genes.

Cell culture

CLL cells were isolated from peripheral blood by density gradient centrifugation, then washed and re-suspended at 3×10^6 cells/mL in RPMI1640 medium (Cambrex, UK) supplemented with 10% fetal bovine serum (Cambrex, UK), non-essential amino acids (Invitrogen, UK), antibiotics (Invitrogen, UK) and Hepes buffer (Cambrex, UK). Isolated CLL cells were then cultured in 6-well plates for 0-6 days. We utilized 30Gy irradiated mouse fibroblast L-cells or L-cells transfected with CD154 (*a gift from Professor J Gordon, Birmingham)* together with IL-4 (R&D Systems) at 20 ng/mL as stromal cell layers to investigate the variability in proliferative response in individual cases of CLL. When indicated, CLL cells were cultured on these stromal cell layers for up to 6 days, with changes of medium on alternate days.

Annexin V assay

The annexin V assay was performed using the BD Annexin V Apoptosis Detection Kit (BD PharMingen: San Diego, CA, USA). CLL cells were harvested, then washed with phosphate-buffered saline (PBS) and re-suspended in 100 μL annexin V binding buffer containing 10 μL fluorescein isothiocyanate (FITC)-conjugated annexin V and 10 μL propidium iodide (PI) for 15 minutes. After this time, an additional 400 μL of annexin V buffer was added and samples analyzed using flow cytometry. Results are displayed as density plots showing annexin V-FITC (FL1) versus PI (FL2) binding.

Intracellular staining

Cells (10^5) were harvested and washed once in PBS. After having been resuspended in 200 μL of FACS staining solution (PBS/1% bovine serum albumin [BSA], 0.05% sodium azide) anti-CD19 PE-Cy5 (BD Biosciences) and anti-CD38 FITC (BD Biosciences) were added and cells were incubated for 30 min on ice. The cells were washed twice in PBS before they were fixed in 2% paraformaldehyde for 2 hours at room temperature. After two washes cells were permeabilized with 200 μL Triton-X (0.05% in PBS) for 20 min at room temperature. An equal volume of 4% BSA in PBS was then added to the cells which were

incubated for a further 30 min at room temperature. Anti-ZAP-70 (clone 2F3.2) (Upstate Biotechnology) at 1 μg/10^5 cells was added and cells were incubated for 40 min on ice. Cell were washed twice in PBS and resuspended in 200 μL of PBS/1% Triton-X/1% BSA and stained with anti-mouse IgG PE (Sigma) as recommended by the manufacturer for 30 min on ice. After two further washes in PBS/0.05% Triton–X, cells were analyzed in a FACScalibur flow cytometer (BD Biosciences) using the CellQuest program. Two-stage gating[28] was used to define CD19-expressing cells without apoptotic cells or cell doublets. In order to establish parameters for the measurement of the percentage of ZAP-70 leukemic B cells we used the internal positive control of ZAP-70 T-cell staining.

Western blots

Protein lysates were prepared and electrophoresis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to standard techniques. After transfer to PVDF membranes staining for ZAP-70 (Abcam, ab18371) and GAPDH (Abcam) was carried out.

[^{3}H] thymidine incorporation assay

CLL cells (10^5) were cultured in 96-well plates on plastic, MSC and γ-irradiated CD40-L and NT-L feeder layers for 5 days, after which time 1 μCi/mL [^{3}H] thymidine (Amersham Biosciences, UK) was added for 16 hours before harvesting and analysis. Incorporated [3H] thymidine was measured using a microplate beta counter and Wallac software.

Results

Regulation of CD38

Surface marker changes associated with CLL cell residence in lymph nodes, rather than in peripheral blood, have been reported and include up-regulation of CD23[29] and CD38.[18] Lymph nodes are also the sites of T-cell containing proliferation centers. The CD40 pathway, stimulated by the T-cell surface marker CD154, provides important proliferative and other signals for normal B cells and we, therefore, tested the effects of the CD154/IL-4 system on CD38 expression. We observed an increase in expression over 5 days that was not seen on culture of CLL cells with NT-L cells or on tissue culture plastic (Figure 1A). A direct comparison of the effects of CD154 and IL-4 showed that the up-regulation of CD38 is almost entirely due to CD154 (Figure 1B). We next investigated whether the effects of the CD154-L/IL-4 system were reversible (Figure 2). After 2 days of culture in the CD154-L/IL-4 system, CLL cells were transferred to NT-L cells, plastic or further CD154-L/IL-4. As anticipated, the CD154-L/IL-4 system increased CD38 expression but surprisingly the NT-L cells also maintained increased expression. We cannot exclude the possibility that contaminating CD154-L cells were the cause of this but following transfer to plastic there was a decline in CD38 expression suggesting that contamination was not a major contributor. CD38 expression varies in CLL and is associated with prognosis.[2,8,30] We, therefore, compared CD38 expression induced by the CD154/IL-4 system with basal CD38 expression (Figure 3). Patients at each clinical stage are able to up-regulate CD38 expression. For patients with clinical stage A CLL, the mean CD38 expression increased from 40% to 53%; for stage B the increase was from 58% to 85%. When analyzed by mutational status, CD38 expression increased from 31% to 68% for mutated cases and from 52% to 73% for unmutated cases. However, there was not a significant difference (Mann-Whitney U-test) between basal and induced CD38 expression. This demonstrates that CD38 expression can be regulated by the CLL cell environment and is not a fixed feature of the malignant clone. Similarly analysis by immunoglobulin gene mutational status showed that cases with mutated immunoglobulin genes up-regulated CD38 expression in the CD154/IL-4 system and again there was not a significant difference between basal and induced expression (Mann-Whitney U-test) (Figure 3B). CD38 is a signaling molecule and can drive proliferation of CLL cells.[12] We, therefore, looked for a correlation between CD38 expression and proliferation as measured by [^{3}H]-thymidine incorporation (Figure 3C). There was no correlation between either basal CD38 expression or expression induced by the CD154/IL-4 system and proliferation, as measured by [^{3}H]-thymidine incorporation (Figure 3C). In addition we showed that high spontaneous apoptosis on plastic is markedly reduced by culture on either CD154/IL-4 or NT-L cells (*Supplementary Figure 1*). These experiments provide evidence that: (i) CD38 expression can be regulated; (ii) stimulation by CD154/IL-4 is sufficient to increase the expression of CD38; (iii) induction is unlikely to be related to clinical stage or prognosis.

The CD154/IL-4 system activates leukemic B cells and causes an increase in cell size associated with proliferation. Flow cytometry (*Supplementary Figure 2*) showed that following culture in the CD154/IL-4 system both the smaller and larger cells had higher CD38 expression than that of leukemic cells cultured on plastic. However, within the CD154/IL-4-stimulated population the larger cells had higher CD38 expression than the smaller cells. Thus, CD38 expression (for each individual patient) is proportional to the degree of cell activation. Whilst an increase in the size of cells alone (rather than true up-regulation of the surface receptor) may contribute to this effect, it is unlikely to account for all the observed increase in expression. For example, we found (in accordance with others,[31] that CD5 expression decreases following CD40 stimulation (*Supplementary Figure 2B*), whilst CD79B does not alter (*Supplementary Figure 2C*), demonstrating that increase in size of cells is not necessarily associated with increase in surface marker expression.

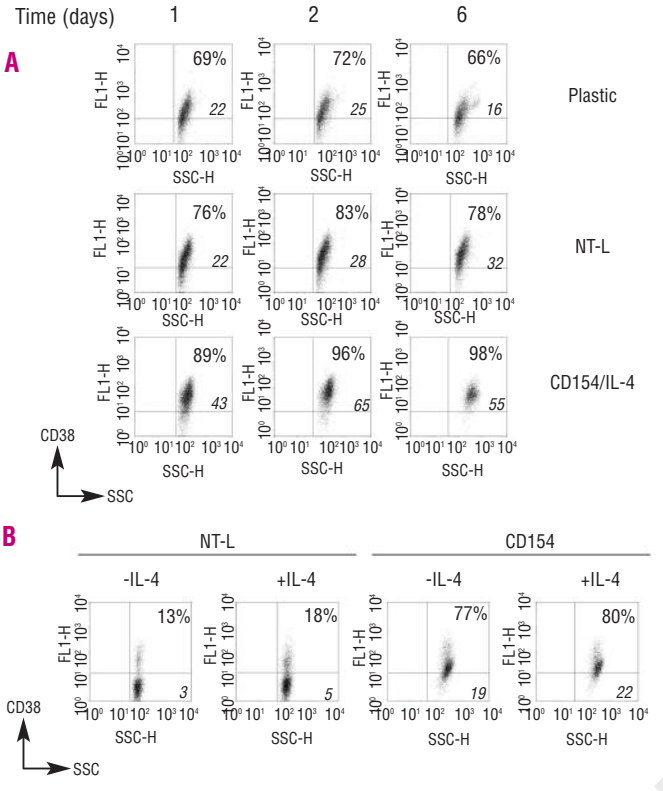

Figure 1. Induction of CD38 expression. **A.** CLL cells were cultured on plastic, NT-L or CD154-L cells/IL-4 for 1, 2 or 6 days. CD38 expression on $CD5^+CD19^+$ B cells was measured by flow cytometry. An isotype control antibody was used to define the $CD38^+$ population. Numbers in the right-hand corner of each dot-plot are the percentages of $CD38^+$ cells. Numbers in italics are the mean fluorescence intensity. Culture on plastic produced no change. NT-L produced an increase in CD38 expression above that seen on plastic. CD154-L cells/IL-4 produced significant increases in CD38 expression at day 1 and further increasesover the time course of the experiment. **B.** To determine the cause of the increase in CD38, CLL cells were cultured with NT-L cells in the presence or absence of IL-4, or with CD154 in the presence or absence of IL-4. CD154 in the absence of IL-4 increased CD38 expression but IL-4 alone had no effect on the fraction of $CD38^+$ cells.

Regulation of CD38 in normal peripheral blood B cells

In order to compare the regulation of CD38 on a normal population of B cells we purified peripheral blood B cells from normal blood donors. A pool from four donors was used in subsequent experiments. Freshly isolated cells showed CD38 expression of about 65% (Figure 4). Over 5 days this level of expression did not change when the cells were cultured on plastic or NT-L, but as with the leukemic cells the CD154-L cells/IL-4 system induced an increased level of expression. We conclude that CD38 is a regulated surface marker in polyclonal populations of normal peripheral blood B cells.

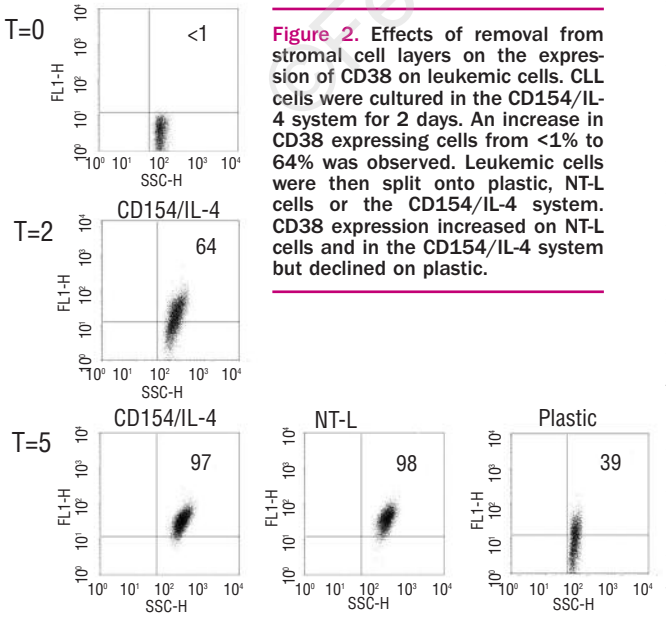

Figure 2. Effects of removal from stromal cell layers on the expression of CD38 on leukemic cells. CLL cells were cultured in the CD154/IL-4 system for 2 days. An increase in CD38 expressing cells from <1% to 64% was observed. Leukemic cells were then split onto plastic, NT-L cells or the CD154/IL-4 system. CD38 expression increased on NT-L cells and in the CD154/IL-4 system but declined on plastic.

Effects of agonistic anti-CD38 antibody

CD38 is a signaling molecule in CLL cells, and an agonistic anti-CD38 antibody (IB4) has been shown to drive proliferation.[12] In order to find out how CD38 signaling altered CD154-L cells/IL-4-stimulated signaling we cultured CLL cells either on plastic, with NT-L cells or in the CD154-L cells/IL-4 system. As previously found, CD154-L cells/IL-4 effectively drove proliferation, but IB4 did not produce an additional increment in [^{3}H]-thymidine incorporation. However, when added to viable CLL cells cultured with NT-L cells and IL-4, IB4 produced a significant (Mann Whitney U-test; $p<0.002$) increase in proliferation in CD38-expressing cases (Figure 7). There was no induction of proliferation when CLL cells were cultured on plastic, and a modest increase only when cultured on NT-L without IL-4. We conclude that the effect of the CD154-L cells/IL-4 system is to drive proliferation at a maximum that is possible *in vitro* and that CD38 signaling cannot increase this further. However, viable but non-proliferating CLL cells, maintained on NT-L cells, did show an increase in proliferation with IB4 suggesting that CD38 signaling may be important in situations in which the leukemic cells are not receiving maximum signals.

Relationship of ZAP-70 expression to CD38 expression

It has been reported that CLL cells co-express CD38 and ZAP-70[32] and also that some of the effects of CD38 signaling may be mediated through ZAP-70 phosphorylation.[33] In order to further define the relationship between CD38 and ZAP-70 we sought to determine whether ZAP-70 expression was induced in parallel with CD38 in our CD154-L/IL-4 system. We stained cells with antibodies to the surface markers CD5, CD19 and CD38 and, after fixation and permeabilization, added anti-ZAP-70 antibody. Electronic gating defined the $CD5^+CD19^+$ cell population and excluded apoptotic cells and cell doublets. ZAP-70 and CD38 expression was measured on this cell population. We investigated those cases with an initial low level of CD38 expression in order to be able to observe the effects of major increases in the level of this marker. In five out of seven cases examined there was an increase in CD38 expression in the CD154/IL-4 system, accompanied by an increase in ZAP-70 expression (Figures 6 C-F). In the cases shown there was an increase in CD38 accompanied by an increase in ZAP-70 and in all the cases analyzed (n=7) (*Supplementary Table 2)* the majority of $ZAP70^+$ cells were also $CD38^+$ (Figures 6C and 6D). However, we also found examples of discordance between induction of $CD38^+$ and $ZAP70^+$. (Figure 8E demonstrates an increase in $CD38^+$ cells without an increase in ZAP-70 expression whereas Figure 8F shows a case with increase in ZAP-70 expression without accompanying increase in CD38). Western blots are shown an accompanying the flow cytometry profiles to confirm these findings. Therefore, we show that there is a dynamic association between the increase in $CD38^+$ cells and expression of ZAP-70 but that this linkage is not invariable. Expression of CD38 and ZAP-70 may be driven by similar stimuli but differences intrinsic to the leukemic cell may in some cases produce discordant expression of these two markers.

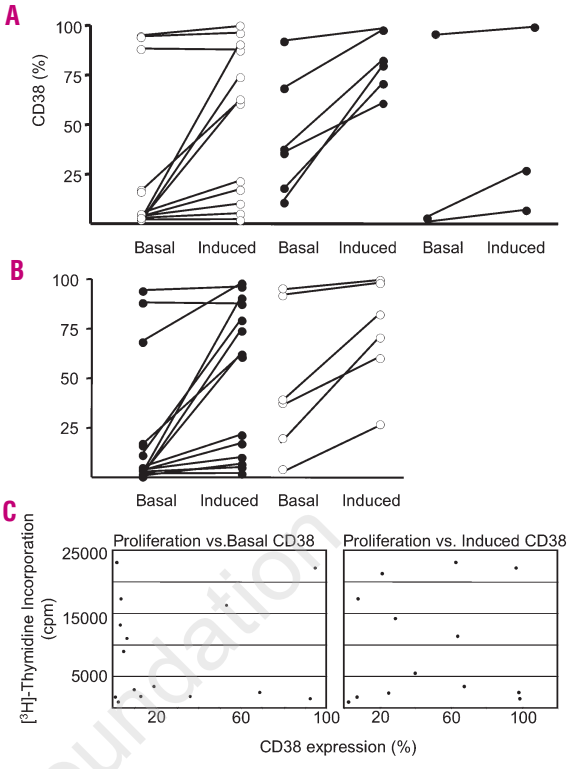

Figure 3.Comparison of CD38 expression induced by CD154-L cells/IL-4 and association between CD38 expression and cell size. Comparison of basal and day 5 induced expression of CD38 from different cases of CLL. (A) Open circles represent samples from patients with clinical stage A, black circles thos with clinical stage B or progressive stage A and grey circles those with stage C. (B) Black circles represent samples from cases with unmutated immunoglobulin genes and open circles from cases with mutated immunoglobulin genes. (C) There is no correlation between either basal or induced expression of CD38 and proliferation as measured by [3H]-thymidine incorporation.

Discussion

CLL cells in lymph nodes have different surface marker and survival protein characteristics from circulating leukemic cells.[18,29,34] Signals contributing to the different pattern of surface marker expression and biological characteristics of CLL cells in the lymph node are not known but are likely to come from follicular dendritic cells and T cells[19] both of which are found in lymph node proliferation centers. CD154 is a T-cell surface molecule delivering essential signals for normal B-cell proliferation in the germinal center. CD154, either as soluble protein or in a cell-membrane bound context with addition of IL-4 is capable of delivering signals to CLL cells.[26,35-38] Systems

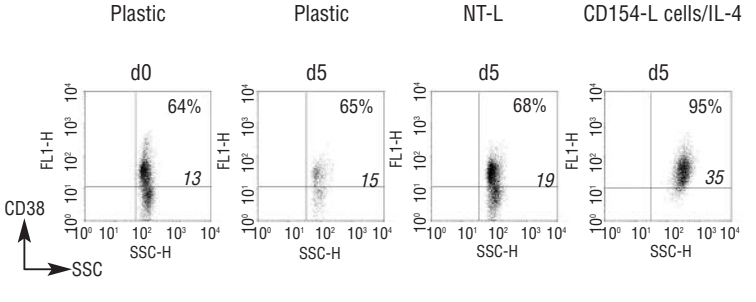

Figure 4. Induction of CD38 in normal B cells. Purified normal peripheral blood B cells were cultured as before for 5 days. The percentages of $CD38^+CD5^+CD19^+$ cells are shown in the top right hand corners and mean fluorescence intensity (MFI) is in italics. There was basal (day 0) expression of CD38 and further up-regulation by CD154-L cells/IL-4 but not by culture on plastic or NT-L.

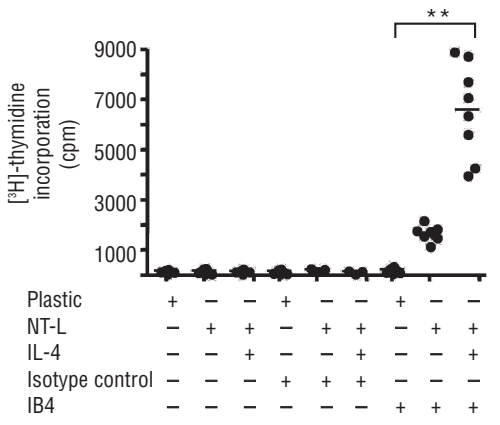

Figure 5 (left). Effect of agonistic anti-CD38-antibody (IB4) on proliferation of CD38 expressing CLL cells. CLL cells were cultured on plastic or NT-L in the presence and absence of 20 ng/mL of IL-4 and either 10 μg/mL of IB4 or 10 μg/mL of a mouse IgG isotype control (BD Biosciences). [3H]-thymidine incorporation was measured in triplicate for seven patients with CD38-expressing CLL cells and the mean values were plotted. The horizontal bar is the mean value for all the patients for each cell culture condition tested. For CLL cells cultured on NT-L, IB4 alone produced a modest increase in thymidine incorporation but when combined with IL-4 there was a statistically significant (Mann Whitney U test; $p<0.002$), increase in proliferation. IB4 was not effective in stimulating proliferation in four CD38- patients (<7% CD5+CD19+ cells). CD154/IL-4 produced [3H]-thymidine incorporation of 26000±4500 cpm. The addition of IB4 did not produce a significant change in [3H]-thymidine incorporation, which was a 22000±7000 cpm.

utilizing CD154 have been shown to alter expression of surface markers, including CD80, CD86 and CD95[38] and Fas[35,37] on CLL cells.

CD38 is a multifunctional surface molecule, with potential roles in both signaling and adhesion,[33] which is expressed on activated B cells including (in humans) ger-

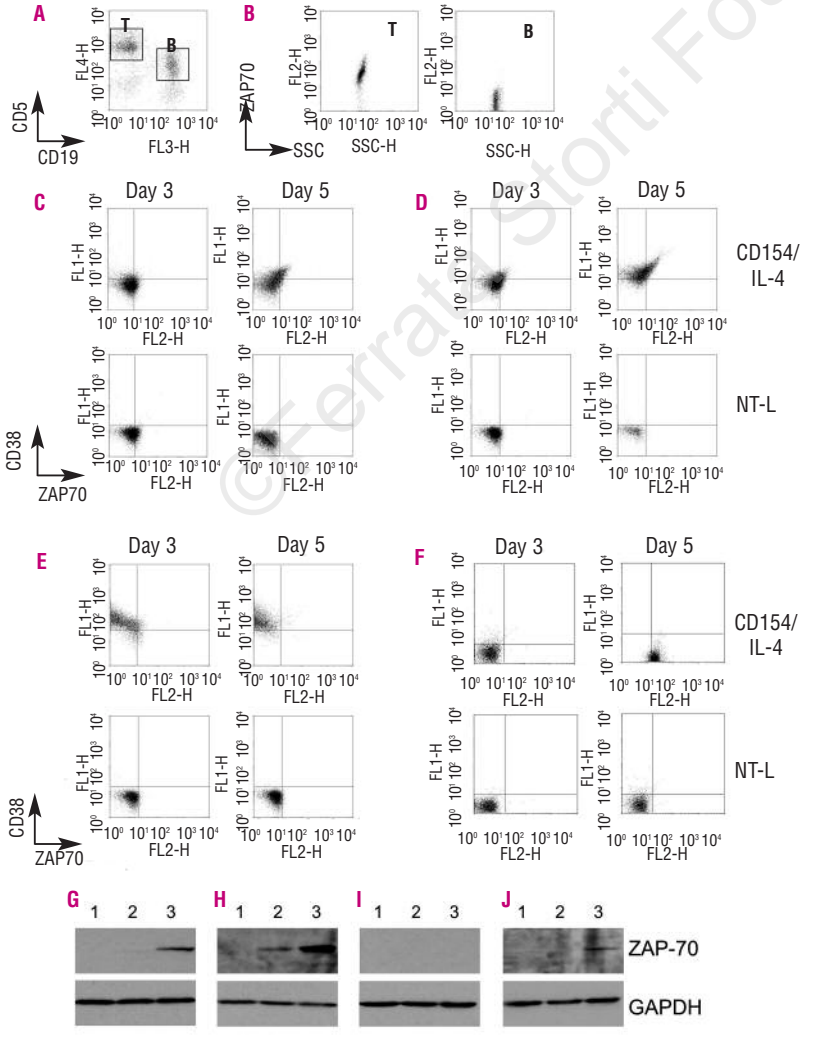

Figure 6. Validation of flow cytometry for detection of ZAP-70. (A) Freshly isolated mononuclear cells from a patient with CLL were stained with anti-CD5, anti-CD19 and anti-ZAP-70. T-cells (T) are defined as CD5+CD19- and the B-cell population (B) as CD5modCD19+. (B) Gating on these two populations demonstrates ZAP-70 expression in T-cells and not leukemic B-cells. CLL cells were cultured for 5 days on NT-L and CD154-L cells/IL-4.
In cases (C) (patient 6) and (D) (patient 1) the CD154/IL-4 system induces both CD38 and ZAP-70. The majority of CD38+ cells co-express ZAP-70. (E) and (F) demonstrate discordant expression of CD38 and ZAP-70. (E) Patient 20 shows an increase in CD38 without induction of ZAP-70 whereas (F) (patient 18) showed induction of ZAP-70 with no change in CD38 expression. (G-J) To confirm the flow cytometry data western blots were carried out. Lane 1 is protein from freshly isolated CLL cells, lane 2 from CLL cells cultured for 5 days with NT-L cells and lane 3 from CLL cells cultured for 5 days in the CD154/IL-4 system. (C) and (G) are from the same patient as are (D) and (H), (E) and (I), and (F) and (J).

minal center B cells. There are differences of opinion on the level of CD38 that best predicts prognosis but high expression on CLL cells is associated with a poor clinical outcome.[2,8,30,39] Activated B cells, including CLL cells, are larger than non-activated cells and CD38 expression correlates with cell size implying that the most activated cells express the highest amounts of this marker.[40] Others have demonstrated that CD38 is induced by IL-2[12] and activated T cells.[41] In this report we show, for the first time, that the T-cell surface marker, CD154, which is likely be a physiological ligand encountered by leukemic B-cells in the lymph node, is sufficient to upregulate CD38. Our data, and previous work,[12] suggest that B-cell activation by a variety of means causes up-regulation of CD38. One hypothesis generated from these data is that CD38 functions are important not only in patients who have high basal expression but also in some cases in which expression can be induced by contact with T cells in lymph nodes.

We observed a wide variation of CD38 responses to CD154-L cells/IL-4. Recently it has been found that polymorphisms at the CD38 locus are related to prognosis in CLL and it is possible that basal or induced expression may be influenced by genetic variation.[42,43]

CD38 is expressed on between 30 and 60% of normal peripheral blood B cells.[44] We confirmed this finding and demonstrated that CD38 expression increases in the CD154/IL-4 system. These findings imply that CD38 expression can be regulated both in polyclonal normal B cells and monoclonal leukemic cell populations. It appears that the fraction of peripheral blood cells, either normal or leukemic, expressing CD38 is a function of how well this population responds to stimulation from CD154 and growth factors in either the germinal center (for normal B cells), or proliferation centers (for CLL cells). Our model postulates that B cells receive signals, in the lymph nodes or bone marrow, which induce CD38 expression and that cases with a poor clinical outcome maintain CD38 expression in the peripheral blood whilst those with a good clinical outcome rapidly lose expression of this surface marker. Our results further show that *high* CD38 expression in CLL is, in fact, similar to the expression observed in normal peripheral blood and suggest that, if considering this surface marker in isolation, cases of CLL that do not express CD38 are abnormal.

ZAP-70 is a prognostic marker in CLL,[4,5] and is also expressed in normal B cells on activation.[45-47] Simultaneous measurements of CD38 and ZAP-70 at single time points and without culture *in vitro* have shown that the expression of these two markers is associated.[32] We have shown that expression of ZAP-70 can be induced together with CD38 expression by the CD154/IL-4 system (*Supplementary Table 2*) in a proportion of patients, although others show discordance in the induction of these two molecules (Figure 8A and 8B). CD40 stimulation, therefore, appears to play a role in driving expression of both CD38 and ZAP-70 but other factors, some of which may be intrinsic to the leukemic cell, also function to regulate the expression of these prognostically important proteins.

Authors' Contributions

SDW designed and carried out experiments, MB designed and carried out experiments, SH carried out experiments, SD designed experiments and wrote the paper and SDW designed experiments, and wrote the paper.

Conflict of Interest

The authors reported no potential conflicts of interest.

References

1. Dohner H, Stilgenbauer S, Benner A, Leupolt E, Krober A, Bullinger L, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. N Engl J Med 2000;343:1910-6.
2. Damle RN, Wasil T, Fais F, Ghiotto F, Valetto A, Allen SL, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood 1999;94:1840-7.
3. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood 1999;94:1848-54.
4. Crespo M, Bosch F, Villamor N, Bellosillo B, Colomer D, Rozman M, et al. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. N Engl J Med 2003; 348:1764-75.
5. Wiestner A, Rosenwald A, Barry TS, Wright G, Davis RE, Henrickson SE, et al. ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. Blood 2003;101:4944-51.
6. Ibrahim S, Keating M, Do KA, O'Brien S, Huh YO, Jilani I, et al. CD38 expression as an important prognostic factor in B-cell chronic lymphocytic leukemia. Blood 2001; 98:181-6.
7. Morabito F, Mangiola M, Oliva B, Stelitano C, Callea V, Deaglio S, et al. Peripheral blood CD38 expression predicts survival in B-cell chronic lymphocytic leukemia. Leuk Res 2001; 25:927-32.
8. Zupo S, Isnardi L, Megna M, Massara R, Malavasi F, Dono M, et al. CD38 expression distinguishes two groups of B-cell chronic lymphocytic leukemias with different responses to anti-IgM antibodies and propensity to apoptosis. Blood 1996; 88:1365-74.
9. Lanham S, Hamblin T, Oscier D, Ibbotson R, Stevenson F, Packham G. Differential signaling via surface IgM is associated with VH gene mutational status and CD38 expression in chronic lymphocytic leukemia. Blood 2003;101:1087-93.
10. Chen L, Apgar J, Huynh L, Dicker F, Giago-McGahan T, Rassenti L, et al. ZAP-70 directly enhances IgM signaling in chronic lymphocytic leukemia. Blood 2005;105:2036-41.
11. Chen L, Widhopf G, Huynh L, Rassenti L, Rai KR, Weiss A, et al. Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia. Blood 2002; 100:4609-14.
12. Deaglio S, Capobianco A, Bergui L, Durig J, Morabito F, Duhrsen U, et al. CD38 is a signaling molecule in B-cell chronic lymphocytic leukemia cells. Blood 2003;102:2146-55.
13. Deaglio S, Vaisitti T, Bergui L, Bonello L, Horenstein AL, Tamagnone L, et al. CD38 and CD100 lead a network of surface receptors relaying positive signals for B-CLL growth and survival. Blood 2005;105:3042-50.
14. Deaglio S, Mallone R, Baj G, Arnulfo A, Surico N, Dianzani U, et al.

CD38/CD31, a receptor/ligand system ruling adhesion and signaling in human leukocytes. Chem Immunol 2000;75:99-120.
15. Deaglio S, Morra M, Mallone R, Ausiello CM, Prager E, Garbarino G, et al. Human CD38 (ADP-ribosyl cyclase) is a counter-receptor of CD31, an Ig superfamily member. J Immunol 1998;160:395-402.
16. Ibrahim S, Jilani I, O'Brien S, Rogers A, Manshouri T, Giles F, et al. Clinical relevance of the expression of the CD31 ligand for CD38 in patients with B-cell chronic lymphocytic leukemia. Cancer 2003; 97: 1914-9.
17. Morabito F, Mangiola M, Stelitano C, Deaglio S, Callea V, Malavasi F. Simultaneous expression of CD38 and its ligand CD31 by chronic lymphocytic leukemia B-cells: anecdotal observation or pathogenetic hypothesis for the clinical outcome? Haematologica 2003;88:354-5.
18. Jaksic O, Paro MM, Kardum Skelin I, Kusec R, Pejsa V, Jaksic B. CD38 on B-cell chronic lymphocytic leukemia cells has higher expression in lymph nodes than in peripheral blood or bone marrow. Blood 2004; 103:1968-9.
19. Caligaris-Cappio F. Role of the microenvironment in chronic lymphocytic leukaemia. Br J Haematol 2003; 123:380-8.
20. Stevenson FK, Caligaris-Cappio F. Chronic lymphocytic leukemia: revelations from the B-cell receptor. Blood 2004;103:4389-95.
21. Burger JA, Tsukada N, Burger M, Zvaifler NJ, Dell'Aquila M, Kipps TJ. Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. Blood 2000;96:2655-63.
22. Pedersen IM, Kitada S, Leoni LM, Zapata JM, Karras JG, Tsukada N, et al. Protection of CLL B cells by a follicular dendritic cell line is dependent on induction of MCL-1. Blood 2002;100:1795-801.
23. Tsukada N, Burger JA, Zvaifler NJ, Kipps TJ. Distinctive features of "nurselike" cells that differentiate in the context of chronic lymphocytic leukemia. Blood 2002;99:1030-7.
24. Banchereau J, de Paoli P, Valle A, Garcia E, Rousset F. Long-term human B cell lines dependent on interleukin-4 and antibody to CD40. Science 1991;251:70-2.
25. Banchereau J, Rousset F. Growing human B lymphocytes in the CD40 system. Nature 1991; 17;353:678-9.
26. Fluckiger AC, Rossi JF, Bussel A, Bryon P, Banchereau J, Defrance T. Responsiveness of chronic lymphocytic leukemia B cells activated via surface Igs or CD40 to B-cell tropic factors. Blood 1992;80:3173-81.
27. Willimott S, Baou M, Huf S, Wagner S. Separate cell culture conditions to promote proliferation or quiescent cell survival in chronic lymphocytic leukaemia. Leuk Lymphoma 2007; 48:1647-50.
28. Rawstron AC, Kennedy B, Evans PA, Davies FE, Richards SJ, Haynes AP, et al. Quantitation of minimal disease levels in chronic lymphocytic leukemia using a sensitive flow cytometric assay improves the prediction of outcome and can be used to optimize therapy. Blood 2001;98:29-35.
29. Lampert IA, Wotherspoon A, Van Noorden S, Hasserjian RP. High expression of CD23 in the proliferation centers of chronic lymphocytic leukemia in lymph nodes and spleen. Hum Pathol 1999;30:648-54.
30. Hamblin TJ, Orchard JA, Ibbotson RE, Davis Z, Thomas PW, Stevenson FK, et al. CD38 expression and immunoglobulin variable region mutations are independent prognostic variables in chronic lymphocytic leukemia, but CD38 expression may vary during the course of the disease. Blood 2002;99:1023-9.
31. Gagro A, McCloskey N, Challa A, Holder M, Grafton G, Pound JD, et al. CD5-positive and CD5-negative human B cells converge to an indistinguishable population on signalling through B-cell receptors and CD40. Immunology 2000; 101:201-9.
32. Pepper C, Brennan P, Alghazal S, Ward R, Pratt G, Starczynski J, et al. CD38+ chronic lymphocytic leukaemia cells co-express high levels of ZAP-70 and are functionally distinct from their CD38- counter-parts. Leukemia 2006;20:743-4.
33. Deaglio S, Vaisitti T, Aydin S, Ferrero E, Malavasi F. In-tandem insight from basic science combined with clinical research: CD38 as both marker and key component of the pathogenetic network underlying chronic lymphocytic leukemia. Blood 2006;108: 1135-44.
34. Smit LA, Hallaert DY, Spijker R, de Goeij B, Jaspers A, Kater AP, et al. Differential Noxa/Mcl-1 balance in peripheral versus lymph node chronic lymphocytic leukemia cells correlates with survival capacity. Blood 2007; 109:1660-8.
35. Dicker F, Kater AP, Fukuda T, Kipps TJ. Fas-ligand (CD178) and TRAIL synergistically induce apoptosis of CD40-activated chronic lymphocytic leukemia B cells. Blood 2005;105: 3193-8.
36. Dicker F, Kater AP, Prada CE, Fukuda T, Castro JE, Sun G, et al. CD154 induces P73 to overcome the resistance to apoptosis of chronic lymphocytic leukemia cells lacking functional P53. Blood 2006; 108:3450-7.
37. Kater AP, Dicker F, Mangiola M, Welsh K, Houghten R, Ostresh J, et al. Inhibitors of XIAP sensitize CD40-activated chronic lymphocytic leukemia cells to CD95-mediated apoptosis. Blood 2005;106:1742-8.
38. Kitada S, Zapata JM, Andreeff M, Reed JC. Bryostatin and CD40-ligand enhance apoptosis resistance and induce expression of cell survival genes in B-cell chronic lymphocytic leukaemia. Br J Haematol 1999; 106:995-1004.
39. Schroers R, Griesinger F, Trumper L, Haase D, Kulle B, Klein-Hitpass L, et al. Combined analysis of ZAP-70 and CD38 expression as a predictor of disease progression in B-cell chronic lymphocytic leukemia. Leukemia 2005; 19:750-8.
40. Manocha S, Matrai Z, Osthoff M, Carter A, Pettitt AR. Correlation between cell size and CD38 expression in chronic lymphocytic leukaemia. Leuk Lymphoma 2003;44: 797-800.
41. Patten P, Buggins A, Richards J, Wotherspoon A, Hamblin T, Mufti G, et al. CD38 expression in B-CLL is dynamic and changes due to contact with activating stimuli found within the leukaemic microenvironment. Blood 2006;[Abstract #2798].
42. Deaglio S, Vaisitti T, Aydin S, Ferrero E, Malavasi F. In-tandem insight from basic science combined with clinical research: CD38 as both marker and key component of the pathogenetic network underlying chronic lymphocytic leukemia. Blood 2006;108: 1135-44.
43. Ferrero E, Saccucci F, Malavasi F. The human CD38 gene: polymorphism, CpG island, and linkage to the CD157 (BST-1) gene. Immunogenetics 1999; 49:597-604.
44. Hoffkes HG, Schmidtke G, Uppenkamp M, Schmucker U. Multiparametric immunophenotyping of B cells in peripheral blood of healthy adults by flow cytometry. Clin Diagn Lab Immunol 1996;3:30-6.
45. Cutrona G, Colombo M, Matis S, Reverberi D, Dono M, Tarantino V, et al. B lymphocytes in humans express ZAP-70 when activated in vivo. Eur J Immunol 2006;36:558-69.
46. Nolz J, Tschumper RC, Pittner BT, Darce J, Kay NE, Jelinek DF. ZAP-70 is expressed by a subset of normal human B-lymphocytes displaying an activated phenotype. Leukemia 2005;19:1018-24.
47. Scielzo C, Camporeale A, Geuna M, Alessio M, Poggi A, Zocchi M, et al. ZAP-70 is expressed by normal and malignant human B-cell subsets of different maturational stage. Leukemia 2006;20:689-95.