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► **To cite this version:**

Andrea Gail Sherman Buggins, Ana Levi, Satyen Gohil, Keith Fishlock, Piers E.M. Patten, et al.. Evidence For A Macromolecular Complex In Poor Prognosis CLL That Contains CD38, CD49d, CD44 and MMP-9.. *British Journal of Haematology*, 2011, 154 (2), pp.216. 10.1111/j.1365-2141.2011.08725.x . hal-00640630

HAL Id: hal-00640630

<https://hal.science/hal-00640630>

Submitted on 14 Nov 2011

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**Evidence For A Macromolecular Complex In Poor Prognosis
CLL That Contains CD38, CD49d, CD44 and MMP-9.**

Journal:	<i>British Journal of Haematology</i>
Manuscript ID:	BJH-2011-00073.R1
Manuscript Type:	Ordinary Papers
Date Submitted by the Author:	31-Mar-2011
Complete List of Authors:	Buggins, Andrea; king's College London, Haematology Levi, Ana; king's College London, Haematology Gohil, Satyen; king's College London, Haematology Fishlock, Keith; King's College Hospital, Haematology Patten, Piers; king's College London, Haematology Calle, Yolanda; king's College London, Haematology Yallop, Deborah; king's College London, Haematology Devereux, Stephen; king's College London, Haematology
Key Words:	CLL IMMUNOLOGY, B LYMPHOCYTES, CHEMOTAXIS

Evidence For A Macromolecular Complex In Poor Prognosis CLL That Contains
CD38, CD49d, CD44 and MMP-9.

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Key words : CLL, CD38, CD49d, CD44, MMP-9

Abstract

Progressive chronic lymphocytic leukaemia is characterised by the accumulation of neoplastic B-cells in the tissues and correlates with the expression of prognostic biomarkers such as CD38, CD49d and matrix metalloproteinase-9 (MMP9), which are involved in migration and tissue invasion. In this study we investigated the physical relationship between these molecules and demonstrate that CD38, CD49d, MMP9 and CD44 are physically associated in a supramolecular cell surface complex. Our findings provide a molecular basis for the correlation between expression of these proteins and prognosis and, since the complex is not present in normal B-cells, suggest a novel leukaemia-specific therapeutic target.

Introduction

Extreme clinical heterogeneity is one of the hallmark features of chronic lymphocytic leukaemia (CLL). Despite the identification of genetic and phenotypic markers that correlate with prognosis, the biological basis of this clinical variability remains unclear. Progressive CLL is defined by expansion of the neoplastic clone and extravascular accumulation in lymphoid tissues, the bone marrow and other organs. Infiltration at these sites gives rise to the characteristic clinical picture of immune dysfunction, lymphadenopathy, splenomegaly and haematopoietic failure; features that correlate with survival (Binet, *et al* 1981, Rai, *et al* 1975). In addition, it is now clear that interactions within the tissues between leukaemic and non-malignant cells provide signals important for maintaining and promoting the growth of the tumour. (Deaglio, *et al* 2005, Edelmann, *et al* 2008, Patten, *et al* 2008)

Given the central role of tissue infiltration in disease progression, it is perhaps unsurprising that many of the biological markers that predict adverse outcome in CLL are linked to processes involved in interaction with the vascular endothelium and/or migration into the extravascular compartment. CD38 (Damle, *et al* 1999) and ZAP-70 (Crespo, *et al* 2003), two of the most frequently cited prognostic markers in CLL, are functionally associated and identify cells with a high migratory potential (Deaglio, *et al* 2007). CD31, the ligand for CD38, is expressed by vascular endothelial cells and participates in the formation of endothelial tight junctions. A number of other molecules involved in migration through endothelium and tissue invasion have also been correlated with disease progression in CLL including CCR7, CD49d and matrix metalloproteinase 9 (MMP-9) (Kamiguti, *et al* 2004, Majid, *et al* 2010). In addition, the membrane of CLL cells has been shown to contain a supramolecular complex involved in tissue migration and metastasis. These structures contain CD49d, CD44 and MMP-9 and are not present in normal B cells (Redondo-Munoz, *et al* 2008). Given the clinical correlation noted between CD38 and CD49d expression in CLL (Majid, *et al* 2010,

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3 Pittner, *et al* 2005), and the known involvement of both in tissue migration, we hypothesised
4 that CD38 might be physically associated with CD49d as part of this supramolecular
5 complex in CLL cells.
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8 9 **Materials and Methods**

10 11 **Samples**

12 Studies correlating CD38 and CD49d expression were performed on 234 consecutive
13 diagnostic samples from patients with confirmed CLL. For immunoprecipitation and
14 immunofluorescence assays, peripheral blood mononuclear cells (PBMCs) were collected
15 and cryopreserved as previously described (Patten, *et al* 2008) from 14 patients with
16 confirmed CLL and known expression of CD38 (Table 1). Ethical approval was obtained
17 from the local institutional review board and informed written consent according to the
18 Declaration of Helsinki.
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26 27 **Flow Cytometry**

28 CD38 and CD49d expression was analysed by 4 colour flow cytometry using anti-CD5, anti-
29 CD19, anti-CD38 and anti-CD49d. For diagnostic assays, antibodies were from Beckman
30 Coulter (High Wycombe, UK) and for assays within individual patients anti-CD5 (Becton
31 Dickinson, Oxford, UK), anti-CD19 (Invitrogen, Paisley, UK), anti-CD38 (eBiosciences,
32 Hatfield, UK) and anti-CD49d (Serotec, Oxford, UK) were used.
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37 38 **Immunoprecipitation**

39 40 CD38

41 For immunoprecipitation studies, a B-cell negative selection procedure was performed on all
42 CLL PBMCs with a CD5+CD19+ percentage of less than 95% of lymphocytes using a B cell
43 enrichment kit without CD43 depletion (Stem cell technologies, UK). Purified cells (1×10^7
44 cells/ml) were then cultured in RPMI supplemented with 10% foetal calf serum, penicillin and
45 streptomycin for 24 hours at 37°C (Complete medium; CM). Suspended cells were collected
46 and lysed for 5 minutes in RIPA buffer (150 mM NaCl, 50 mM tris HCL pH 7.4, 1mM EDTA,
47 5 mM EGTA, 5 mM sodium molybdate, 20 mM phenylphosphate, 1% sodium deoxycholate,
48 0.1% SDS, 1% triton x 100 + phosphatase inhibitors [1mM sodium orthovanadate, 50 mM
49 sodium fluoride] + proteinase inhibitors [1 mM PMSF, 10 ug/ml aprotinin, 20 ug/ml
50 leupeptin] + 2% BSA). Lysates were then centrifuged and the supernatants diluted 100 fold
51 in 50mM tris buffer with 2% BSA pH 7.5. These were then pre-cleared for 2h at 4°C with 40
52 µg of an irrelevant mouse monoclonal IgG (Sigma Immunochemicals, Gillingham, UK) after
53 which 25 µg of Immunopure protein A/G agarose beads (AbCam, Cambridge UK) were
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3 added and incubated for 1 hour at 4°C and then pelleted. The supernatants containing
4 proteins of interest were removed and split into two; half was immunoprecipitated with 20 µg
5 of mouse monoclonal IgG to human CD38 (AbCam, clone no. AT13/5) and half with 20 µg of
6 irrelevant mouse monoclonal IgG after which 25 µl of protein A/G beads were added for 2h
7 at 4°C. The mixtures were then centrifuged and the pellets were washed x1 in RIPA buffer
8 with 2% BSA, followed by a further wash with NP-40 buffer containing 10 mM tris, pH 7.4, 1
9 mM, 1mM EDTA, 150 Mm NaCl, 1% triton x 100 and 0.2 mM sodium orthovanadate, one
10 wash with 50 mM tris EDTA, and one with 200 mM NaCl. The solid phase was left in the
11 wash buffer for 10 mins at each wash and the pellet was transferred to a fresh tube prior to
12 the last wash. The pellets were re-suspended in reducing 2x Laemelli buffer (125mM tris
13 HCL pH 6.8, 4% SDS, 40% glycerol, 200mM dithiothreitol [DTT], bromophenol blue) and
14 heated at 100°C for 5 mins on a heat block.
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23 CD49d

24 For CD49d immunoprecipitation, polyclonal rabbit immunoglobulin (Ig) (sc-1840, Santa Cruz,
25 CA, USA) was used to pre-clear the lysates and anti-CD49d (clone H-210, Santa Cruz) was
26 used to isolate the CD49d-interacting protein complex. The rest of the procedure was
27 identical to the CD38 immunoprecipitation except the reducing agent (DTT) was omitted
28 from the Laemelli buffer. In the absence of DTT, the Ig species remain intact and can be
29 differentiated from CD38 which otherwise runs in the same position on an SDS gel as the Ig
30 heavy chain. For CD38, CD49d, MMP-9 and CD44 positive controls, Daudi B-cells were
31 solubilised in RIPA buffer.
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39 Western Blotting

40 Western blotting was performed using the Novex Powerease system (Invitrogen, Paisley,
41 UK) and the immuno-complex resolved using NuPAGE 4-12% bis-tris gels (Invitrogen) as
42 per the manufacturer's instructions and then transferred to nitrocellulose membranes,
43 blocked with 5% non fat milk and 0.05% tween 20/ PBS for 30 mins then incubated with
44 primary CD44, CD49d, MMP-9 (Abcam, clones EPR1013Y, EPR1355Y and EP1254
45 respectively) or CD38 (R&D Systems, clone 240726). This was followed by incubation for 1h
46 at room temperature with HRP-labelled secondary antibodies (goat anti-mouse at 1:100 or
47 goat anti-rabbit at 1:50 [Dako, UK]). The immunoblots were washed x3 in 0.05% Tween 20/
48 PBS and developed using the enhanced chemiluminescent detection method (ECL plus GE
49 Healthcare, Hitchin, Buckinghamshire). A pre-stained protein marker was used to assign
50 molecular weight to the isolated proteins (Invitrogen).
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Immunofluorescence Microscopy

CLL cells were seeded on poly-L-lysine coated glass slides at 1×10^6 cells/ml in CM and incubated in damp chambers at 37°C for 24 hours, after which the excess medium was removed via suction. Cells were fixed in 1% paraformaldehyde at room temperature for 15 mins, then washed x3 with PBS, permeabilised with 0.5% triton x100 for 10 mins and subsequently washed in phosphate buffered saline (PBS) x3, before blocking with 10% donkey serum (Sigma) / PBS for 30 mins. This was removed under suction and cells incubated overnight at 4°C with primary antibody. After washing, cells were blocked once again with 10% donkey serum/ 4% BSA in PBS for 30 mins, washed again and incubated for 2 hours in secondary antibodies. Details of antibodies used are given in table 2. Slides were washed and stained with DAPI, washed and fixed with anti-fade mounting solution (Invitrogen) and coverslips applied. Images were acquired using a Leica TCS-13000 DBS inverted epifluorescence confocal microscope with x63 and x100 oil immersion objective.

Results

Correlation of CD38 and CD49d expression.

We investigated the relationship between CD38 and CD49d expression using diagnostic samples. Peripheral blood samples from 234 patients with a confirmed diagnosis of CLL were analysed using 4 colour flow cytometry and CD38 and CD49d expression quantified on the CD19+/CD5+ cells. A correlation was observed between both the percentage positivity (Figure 1A) and mean fluorescent intensity (MFI) of CD38 and CD49d expression ($r^2=0.43$, $p<0.0001$, $n=234$; $r^2=0.28$, $p<0.0001$, $n=61$ respectively). In addition to the between patient relationship a correlation between CD38 and CD49d levels **on cells** within individual patients was also observed. **To illustrate this CD5+CD19+ cells were gated into 15 subsets according to their CD38 expression (gates 1-15) and the mean CD49d fluorescence within each of the 15 gates was plotted against the mean CD38 level.** In each of 6 cases analysed there was a statistically significant relationship between CD38 and CD49d levels. Figure 1B illustrates the gating strategy **applied to a representative experiment.**

Co-localisation of CD38 with CD49d, CD44 and MMP-9.

PBMCs from patients with CLL were seeded onto poly-L-lysine slides and stained for CD38 and each of CD49d, CD44 and MMP-9. Slides were analysed using multi-parameter confocal immunofluorescence microscopy. We examined 6 patients for CD38/CD49d co-localisation and 3 patients for co-localisation of CD38 with CD44 and MMP-9. All patients tested demonstrated regions of co-localisation of CD38 with CD49d, CD44 and MMP-9

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3 (Figure 2). **As a negative control, we labelled CLL cells with CD5, CD38 and CD49d.**
4 **Whilst CD38 and CD49d clearly colocalise, CD5 does not (Figure 3).**
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7 **CD49d, CD44 and MMP-9 co-immunoprecipitate with CD38 in CLL cells**

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9 We next tested whether CD38 interacted with the previously described complex involved in
10 cell migration containing CD49d, CD44 and MMP-9. CD38 immunoprecipitations from 11 out
11 of 14 patients contained CD49d, 12 out of 14 CD44 and 9 out of 14 MMP-9. When
12 immunoprecipitates were performed with CD49d, 3 out of 3 patients contained CD38.
13 Representative blots are shown in Figure 4. All assays were controlled using an irrelevant
14 IgG control antibody, confirming the specificity of the findings.
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19 **Discussion**

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21 In this study we have show that the level of CD38 and CD49d expression are strongly
22 correlated in CLL, both within and between patients. We provide convincing evidence that
23 the CLL cell surface membrane contains a preformed complex in which CD38 is physically
24 associated with CD49d, CD44 and MMP9. Although the biological consequences of this
25 finding remain to be defined, these molecules share a number of important clinical and
26 functional features that suggest the complex plays a central role in the biology of progressive
27 CLL and may be an important therapeutic target.
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34 CD38 is a multifunctional membrane protein whose level of expression varies during B cell
35 differentiation. Its expression on CLL cells is dynamic (Patten, *et al* 2008) and ligation, by
36 agonistic antibody (Deaglio, *et al* 2003) or fibroblasts transfected with its ligand CD31 leads
37 to a calcium flux, increased proliferation and improved viability (Dr C. Pepper University of
38 Cardiff, personal communication, Dec. 2010). It has thus been proposed that in-vivo
39 interactions between CD38 positive CLL cells and endothelial CD31 generates signals that
40 promote tumour viability and proliferation. This may in part explain why high expression of
41 CD38 by the tumour cells predicts for adverse outcome in CLL. In addition to its role in
42 promoting survival and proliferation, CD38 has also been reported to be involved in the
43 migration of CLL cells into the tissues. Expression of CD38 and ZAP-70 identifies CLL cells
44 with enhanced migration to the chemokine CXCL12 (Deaglio, *et al* 2007) and a functional
45 link between the two has thus been proposed.
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54 The migration of lymphocytes into tissues is initiated by chemokine induced activation of
55 integrins, which increases their affinity for endothelial ligands such as ICAM1 and VCAM1
56 (Worthylake and Burridge 2001). This results in firm adhesion and migration though the
57 endothelial barrier. In some cells integrins are arranged in supramolecular complexes
58 termed podosomes (Calle, *et al* 2006) that are linked to the cytoskeleton and contain other
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3 proteins involved in tissue invasion such as matrix metalloproteinases. Recent evidence
4 suggests that CLL cells, but not normal B cells, possess podosomes containing $\alpha 4\beta 1$
5 integrin (VLA4 - of which CD49d is the α subunit), a splice variant of CD44 (CD44v) and
6 MMP-9 (Redondo-Munoz, *et al* 2008). CD44 is a proteoglycan that binds to hyaluronic acid
7 whilst MMP-9 is proteolytic proenzyme involved in degradation of the extracellular matrix.
8 Interestingly, the level of expression of CD44v (Zarcone, *et al* 1998), CD49d (Kamiguti, *et al*
9 2004, Majid, *et al* 2010, Shanafelt, *et al* 2008, Tenca, *et al* 2003, Till, *et al* 2002) and MMP9
10 (Kamiguti, *et al* 2004) have all been linked to the presence of bulky lymphadenopathy and/or
11 adverse outcome in CLL.
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19 In addition to their role in tissue invasion, interactions between VLA4 and its ligand VCAM1
20 have also been shown to promote the viability of CLL cells and high levels of CD49d are
21 associated with increased numbers of VCAM1 expressing microvessels within lymph nodes
22 (Zucchetto, *et al* 2009). These observations are paralleled by work from our group showing
23 that CD38 positive cases of CLL have increased numbers of microvessels in lymph nodes
24 (Patten, *et al* 2008) and a more recent study in which interactions with vascular endothelial
25 cells were shown to inhibit CLL cell apoptosis through NF κ B mediated regulation of Bcl-2
26 family proteins (Buggins, *et al* 2010). Incubation with endothelial cells also resulted in NF κ B
27 dependent up-regulation of CD38 and CD49d at both the protein and transcription level.
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35 It is thus abundantly clear that CD38 and CD49d share numerous features in CLL. They are
36 co-ordinately regulated by NF κ B activation, mediate pro-survival signalling and migration
37 into the tissues, are associated with increased lymph node vascularity, predict adverse
38 outcome and their expression levels are strongly correlated both between and within
39 individual patients with CLL. The findings of the present study provide a structural basis for
40 these striking observations. Given the known properties of the individual molecules and their
41 functional overlap it seems very likely that the CD38/CD49d/MMP9/CD44 complex plays a
42 role in migration into the tissues and in pro-survival signalling. Accumulation of leukaemic
43 cells in the tissues is responsible for many of the clinical features of CLL and defines more
44 advanced and progressive disease with an adverse outcome (Binet, *et al* 1981, Rai, *et al*
45 1975). Tissue invasion also exposes the tumour cells to other components of the leukaemic
46 microenvironment such as T cells, nurse-like cells and other stromal elements that promote
47 expansion of the leukaemic clone (Buggins, *et al* 2010, Burger, *et al* 2000, Caligaris-Cappio
48 2003, Patten, *et al* 2008). The identification of the CD38/CD49d complex thus suggests a
49 biological basis for the correlation between expression of these molecules and prognosis.
50 Since the structure is not present in normal B cells, it also presents a novel leukaemia-
51 specific target for the therapy of this common disorder.
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Table 1

Patient ID	Mutational status	Peripheral blood CD38 (%)	Cytogenetics/FISH	Stage
1	M	65	Normal	C
2	M	18	11q-, 13q-	B
3	U	85	8p+, 11q-, 17p-	B
4	M	83	12+	A
5	N/A	10	N/A.	A
6	U	88	11q-, 14q-, 17p-	A
7	M	94 bimodal	17p-	B
8	U	26	17p-, complex	B
9	U	60	11q-, 13q-, 17p-	B
10	U	48	13q-	B
11	U biclonal	19 bimodal	11q-, 17p-	C
12	M	18	12+	A
13	N/A	44	Normal	B
14	U	94	t(2;14), 12+	C

Characteristics of the 14 patients under study.

U= Unmutated, M= Mutated, N/A = Not tested or non-identifiable clones.

Table 2

Primary			Secondary		
Specificity	Species	Source	Specificity	Source	Fluorochrome
CD5	M (m)	CRIS1, SantaCruz	D α M	Jackson	Alexa 488
CD38	Rt (m)	sc90, S-C	D α Rt	Jackson	Cy5
CD44	Rb (m)	EPR1013Y, Abcam	D α Rb	Jackson	Alexa 488
CD49d	Rb (m)	EPR1355Y, Abcam	G α Rb	Jackson	FITC
			D α Rb	Jackson	Cy3*
MMP9	Rb (m)	EP1254, Abcam	D α Rb	Jackson	Alexa 488

Abbreviations: M=Mouse, G=Goat, Rt=Rat, Rb=Rabbit, D=Donkey, (m)=monoclonal, (p)=polyclonal.

Antibodies used for immunofluorescence staining of CLL cells. * indicate fluorochrome used to stain CD49d when co-staining for CD5 and CD38.

Figure legends

Figure 1.

There is a statistically significant correlation of CD38 and CD49d expression in patients with CLL.

A. The plot compares the percentage CD38 and CD49 expression on CD5+CD19+ cells derived from 234 CLL patient samples. Correlation of CD38 and CD49d expression is highly statistically significant (Pearson's correlation)

B. This figure shows that, within an individual patient, levels of CD38 and CD49d expression correlate with each other. Fifteen subsets of CD5+CD19+ cells were created, defined by increasing CD38 expression (diagrammatic example shown on left hand panel). **Mean CD49d MFI for the cells within each of the 15 gates was established and correlated with mean CD38 MFI within the same gate.** A representative patient is shown demonstrating a statistically significant correlation between CD38 and CD49d MFI.

Figure 2.

There is co-localisation of CD38 with CD49d, CD44 and MMP-9.

Representative immunofluorescence microscopy demonstrating staining of CLL cells with CD38 (red) and CD49d, CD44 and MMP-9 (all green). Co localisation is shown in orange/yellow. Two different patterns of CD38/CD49d staining were seen, one showing condensed staining and the other a more diffuse pattern.

Figure 3.

CD5 does not co-localise with CD38 and CD49d.

Three colour immunofluorescence microscopy demonstrating staining of CLL cells with CD49d (blue), CD38 (red) or CD5 (green). Co localisation of CD38 and CD49d is shown in pink in the overlay. CD5 has a distinctly different pattern of expression and remains green in the overlay.

Figure 4.

CD38 immunoprecipitates from CLL cells contain CD49d, CD44, MMP-9 whilst CD49d immunoprecipitates contain CD38.

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3 **A.** Representative blot demonstrating the presence of CD38 (45kda) in a CD49d IP.
4 Irrelevant IgG IP was performed as a negative control. A Daudi cell lysate was probed with
5 α CD38 as a positive control.
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9 **B.** Representative blots demonstrating the presence of (i) CD49d, (ii) CD44 and (iii) MMP-9
10 in a CD38 IP. A Daudi cell lysate was probed with α CD49d, α CD44 and α MMP9 as a
11 positive control .
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For Peer Review

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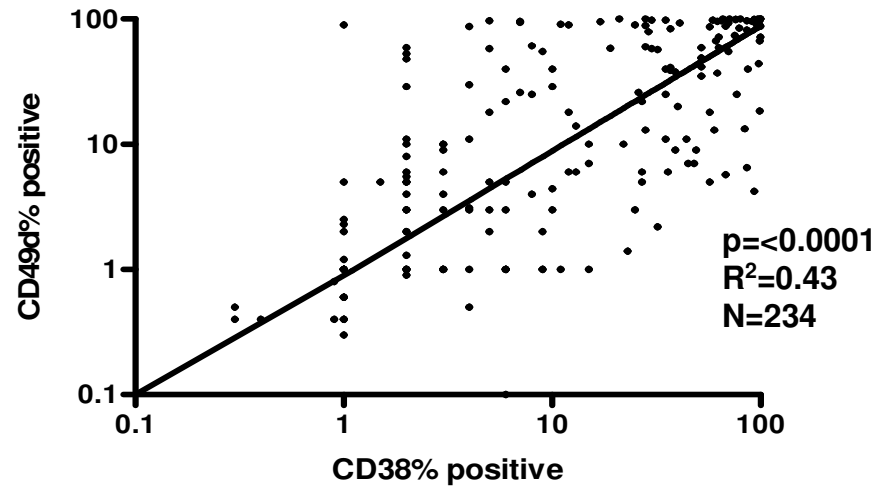
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Zucchetto, A., et al. (2009) CD38/CD31, the CCL3 and CCL4 chemokines, and CD49d/vascular cell adhesion molecule-1 are interchained by sequential events sustaining chronic lymphocytic leukemia cell survival. *Cancer Res*, **69**, 4001-4009.

For Peer Review

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B.

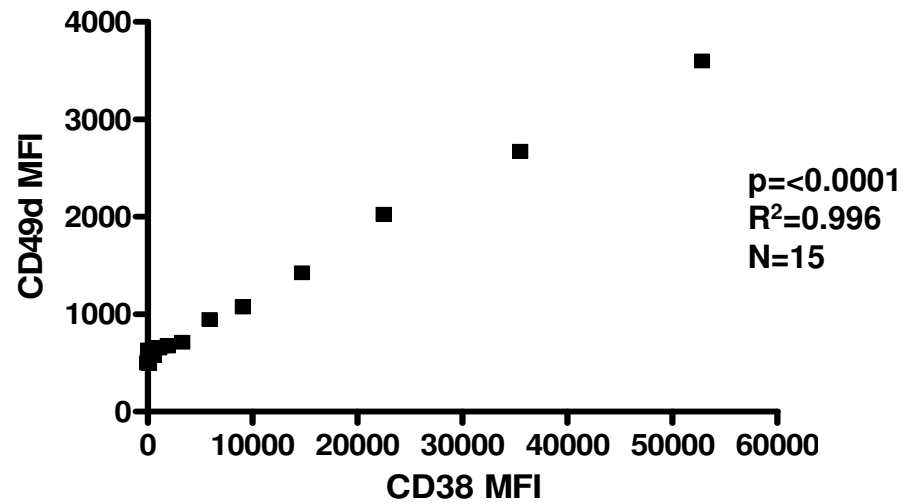
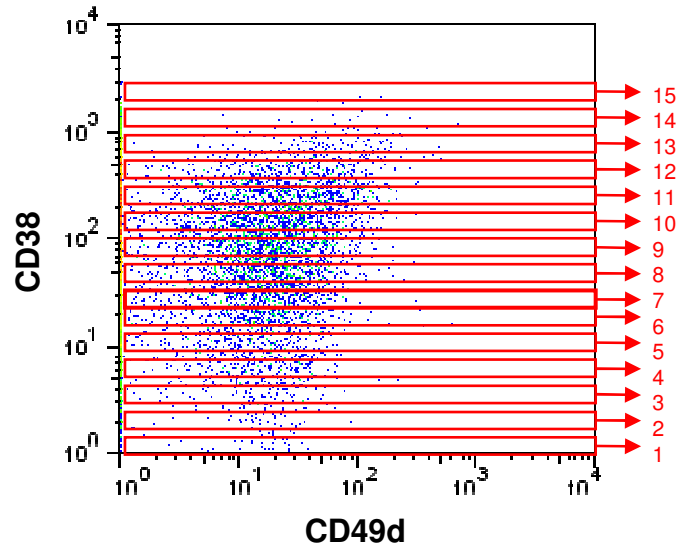


Figure 1

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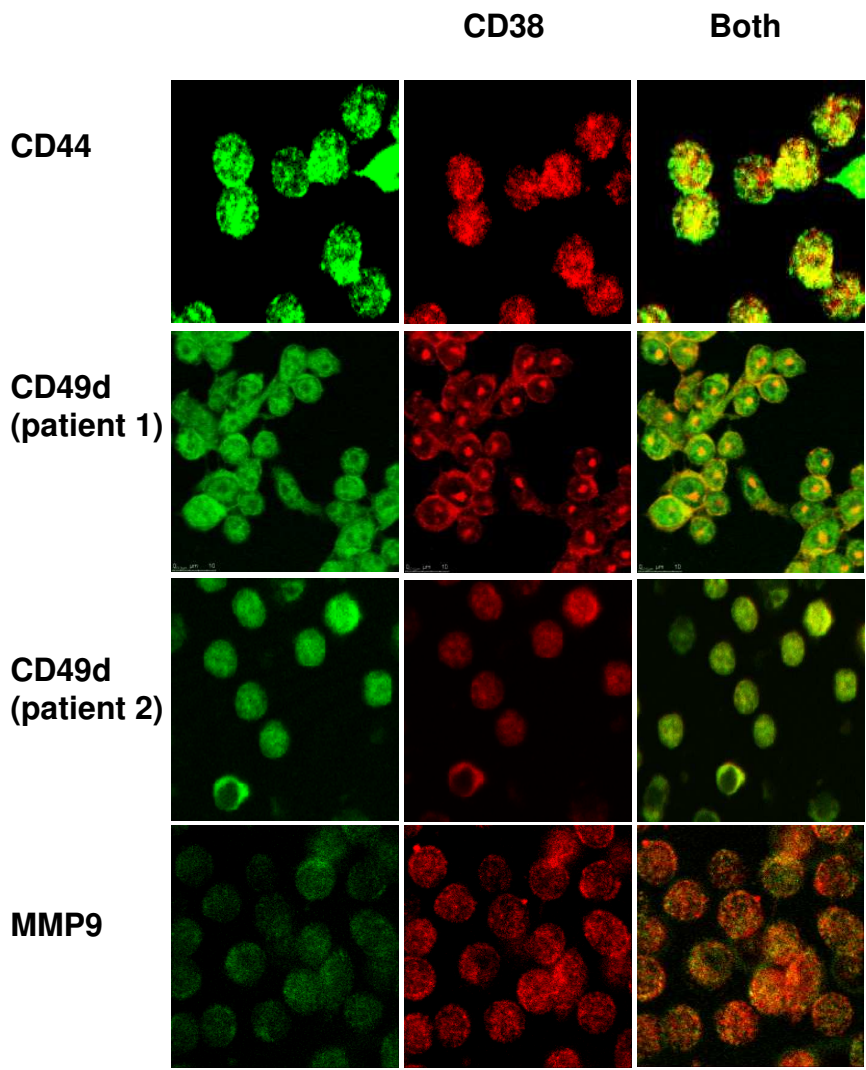


Figure 2

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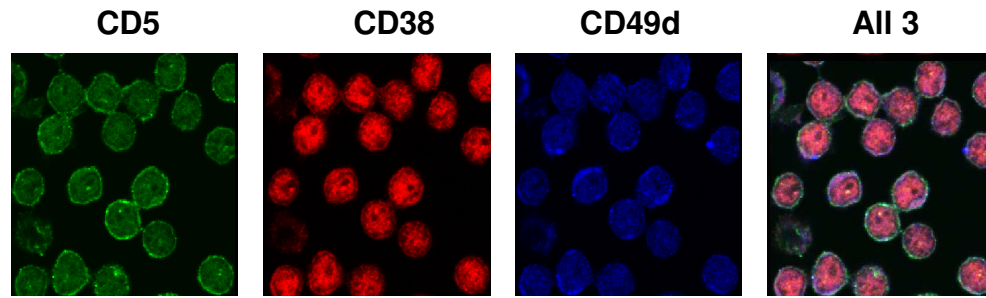


Figure 3

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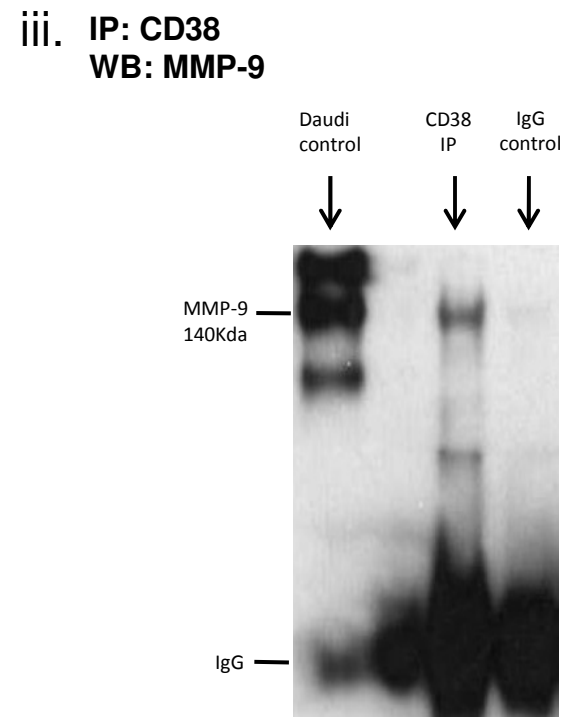
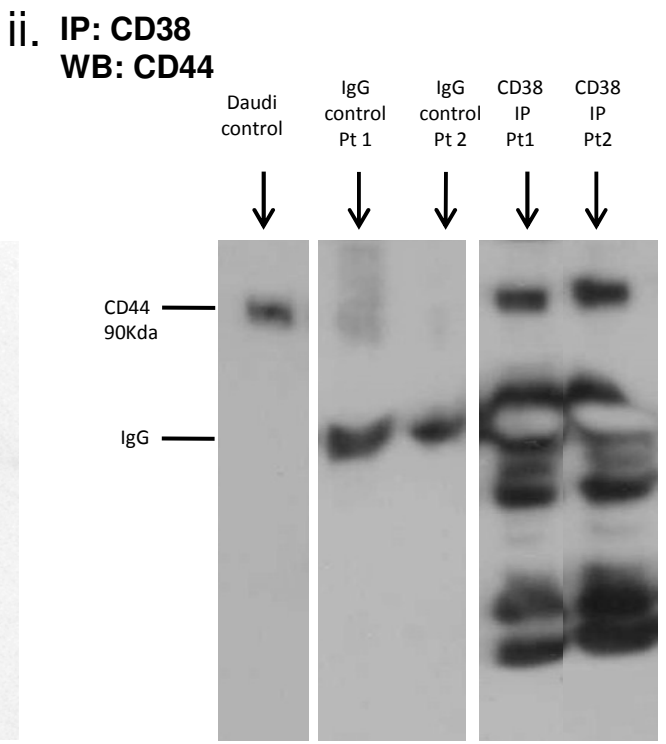
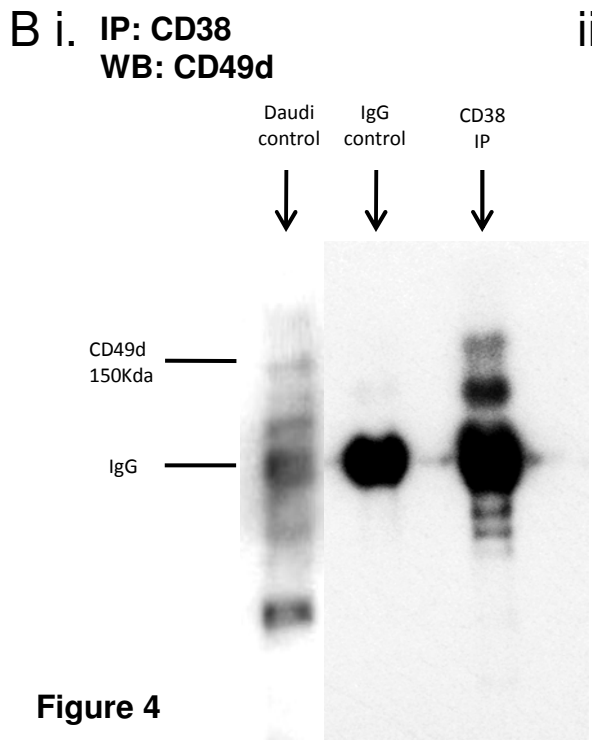
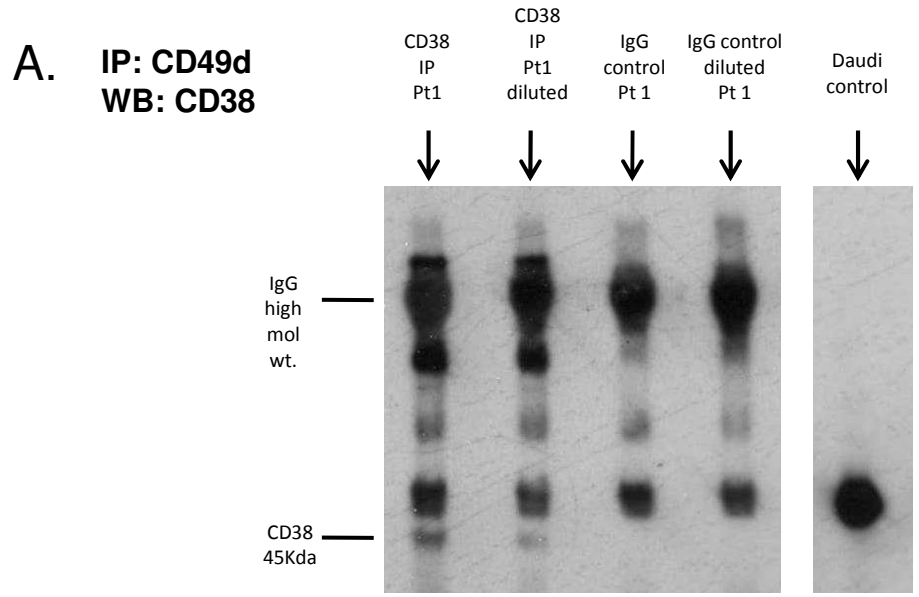


Figure 4