

## ACCEPTED ARTICLE PREVIEW

Accepted Article Preview: Published ahead of advance online publication

**Nurse-like cells control the activity of chronic lymphocytic leukemia B cells via galectin-1**

DO Croci, PE Morande, SD Dylon, M Borge, MA Toscano, JC Stupirski, RF Bezares, JS Avalos, M Narbaitz, R Gamberale, GA Rabinovich, M Giordano

**Cite this article as:** DO Croci, PE Morande, SD Dylon, M Borge, MA Toscano, JC Stupirski, RF Bezares, JS Avalos, M Narbaitz, R Gamberale, GA Rabinovich, M Giordano, Nurse-like cells control the activity of chronic lymphocytic leukemia B cells via galectin-1, *Leukemia* accepted article preview 7 November 2012; doi: [10.1038/leu.2012.315](https://doi.org/10.1038/leu.2012.315).

This is a PDF file of an unedited peer-reviewed manuscript that has been accepted for publication. NPG are providing this early version of the manuscript as a service to our customers. The manuscript will undergo copyediting, typesetting and a proof review before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers apply.

Accepted article preview online 7 November 2012

1 **Nurse-like cells control the activity of chronic lymphocytic leukemia B**  
2 **cells via galectin-1**

3 Letter to the Editor

4

5 Chronic lymphocytic leukemia (CLL) cells are preferentially activated in so-called  
6 proliferation centers frequently found in lymph nodes and bone marrow from CLL patients<sup>1</sup>.  
7 In these “privileged” sites leukemic cells establish close contact with a variety of cell types  
8 that provide long-term support for their survival and progression. In addition, CLL cells  
9 favor the establishment of immunosuppressive microenvironments by altering the cytokine  
10 milieu<sup>2</sup>. Galectin 1 (Gal1), an endogenous  $\beta$ -galactoside-binding lectin found at sites of  
11 inflammation and tumor growth, displays pro-survival activity on malignant cells as  
12 demonstrated for CD45RA(-) primary myeloma cells<sup>3</sup>. Moreover it controls tumor cell  
13 proliferation and invasiveness and plays key roles in tumor-immune escape by dampening T  
14 cell-mediated immunity<sup>4</sup>. In Hodgkin lymphoma Gal1 is over-expressed in Reed-Sternberg  
15 cells, is a predictive biomarker of disease progression and is responsible for creating the  
16 Th2/regulatory T cell-skewed microenvironment typical of this lymphoproliferative  
17 disease<sup>5</sup>. These unique characteristics of Gal1 prompted us to investigate its potential role in  
18 CLL biology.

19

20 We first assessed the expression of Gal1 in peripheral blood and bone marrow  
21 samples from CLL patients using qRT-PCR, flow cytometry and immunohistochemistry.  
22 We found that monocytes in peripheral blood (Fig 1.A and B) and stromal and myeloid cells  
23 in bone marrow biopsies (Fig 1.C) are the main sources of Gal1. CLL cells do not express  
24 Gal1, but they are able to bind Gal1 in a dose-dependent manner (Fig 1.D). This effect was  
25 glycan-specific, as addition of the disaccharide lactose, but not sucrose inhibited binding of  
26 Gal1 to CLL cells (Fig 1.D).

27 In the presence of leukemic cells, monocytes from CLL patients can differentiate *in*  
28 *vitro* into large, adherent cells that protect the leukemic clone from spontaneous and drug-  
29 induced apoptosis. These so-called nurse-like cells (NLC) reside in lymphoid tissues where  
30 they presumably deliver pro-survival and stimulating signals to CLL cells<sup>6</sup>. To determine  
31 whether Gal1 secreted by myeloid cells can influence leukemic B cells responses, we  
32 knocked down Gal1 synthesis in NLC. For this purpose, we differentiated NLC from  
33 peripheral blood CLL samples as previously described<sup>7</sup>, removed non-adherent cells (> 90%  
34 leukemic B cells) and transduced adherent NLC with retrovirus expressing Gal1-specific  
35 short hairpin RNA (shRNA-gal1) or a scrambled control shRNA (shRNA-scr) (Fig 1.E, F).  
36 After 6 h of incubation, transduced NLC were thoroughly washed and non-adherent cells  
37 were incorporated to the plates for further co-culture. Thereafter, we evaluated in the  
38 leukemic clone expression of the activation markers CD80, CD86 and CD25, production of  
39 IL-10 as a prototypical anti-inflammatory cytokine and synthesis of CCL3 and CCL4 as key  
40 chemokines responsible for the recruitment of monocytes and T lymphocytes to lymphoid  
41 tissues. We found that blockade of Gal1 in NLC impaired the expression of activation  
42 markers in CLL cells suggesting that the presence of endogenous Gal1 in myeloid cells is  
43 required for full stimulation of the leukemic clone (Suppl. Fig 1). Blockade of Gal1 also  
44 decreased mRNA and protein levels of IL-10 and mRNA levels of CCL3 in CLL cells,  
45 without affecting those of CCL4 (Fig 1.G, H). While previous reports showed that  
46 recombinant Gal1 induces the release of IL-10 from activated T lymphocytes and dendritic  
47 cells<sup>8</sup>, there is still no information on its effects on CCL3. Of note, both IL-10 and CCL3,  
48 are relevant in CLL pathogenesis as their serum concentrations are elevated in CLL  
49 patients<sup>9,10</sup> and, more importantly, they correlate with shorter time-to-first treatment (TTFT)  
50 and survival<sup>11</sup>.

51 Since B-cell receptor (BCR) signaling plays a central role in the survival,  
52 proliferation and trafficking of CLL cells<sup>12</sup>, we evaluated whether Gal1 can modulate this

53 pathway. We found that, in the presence of Gal1, suboptimal concentrations of anti-IgM can  
54 fully activate the BCR signaling in CLL cells, as assessed by Syk and Erk1/2  
55 phosphorylation (Fig 1.I), indicating that Gal1 may decrease the threshold of BCR  
56 activation probably through the formation of lattices as previously suggested for the pre-  
57 BCR synapse formation and other receptor-ligand systems<sup>4, 13</sup>. While these results suggest  
58 that Gal1 secreted by NLC may exert a direct effect on the leukemic clone, we also found  
59 that knocking down Gal1 diminished the expression of BAFF and APRIL in NLC (Fig 1.J).  
60 Although not evaluated in leukemic cells, BAFF is able to enhance CD86 and induce the  
61 secretion of IL-10 in resting B cells<sup>14</sup>. Hence, Gal1 secreted by NLC might directly or  
62 indirectly influence CLL activity through glycan-dependent binding to these cells and  
63 modulation of BCR signaling or through the control of BAFF and/or APRIL secretion.

64 Next, we determined the concentration of Gal1 in plasma from 49 CLL samples and  
65 40 age-matched healthy donors. Clinical features of CLL patients are depicted in  
66 Supplementary Table 1. Plasma concentrations of Gal1 were significantly increased in CLL  
67 patients compared to healthy subjects (Fig. 2.A;  $p < 0.0001$ ). When we discriminated CLL  
68 patients in high and low risk groups according to the expression of CD38 and ZAP-70 on  
69 CLL cells, we observed a trend (although not statistically significant) to increased levels of  
70 Gal1 in plasma from patients expressing one or both prognostic markers compared to the  
71 double-negative group (Fig.2.B). Similarly, we observed that patients in Binet A staging had  
72 about half the concentration of Gal1 in plasma compared to patients in Binet C (208 vs 517  
73 ng/ml,  $n=25$  vs 7). Finally, we analyzed the expression of Gal1 in bone marrow biopsies  
74 from patients with stable and progressive disease. We found both an increased number of  
75 cells expressing Gal1 and a higher expression of this lectin in bone marrow samples from  
76 patients with progressive disease (Fig. 2.C, E). In agreement with previous reports<sup>15</sup>, we  
77 also observed increased numbers of CD68<sup>+</sup> cells in samples from patients with progressive  
78 disease (Fig. 2D, E). These data indicate that Gal1 is associated with poor outcome in CLL.

79 Collectively, our findings suggest that Gal1 secreted by accompanying myeloid cells  
80 (i.e. NLC, macrophages and dendritic cells) contributes to stimulate the activity of CLL  
81 cells and may help to establish the appropriate microenvironmental conditions for leukemic  
82 progression. From a therapeutic standpoint, our study suggests that selective manipulation  
83 of Gal1 expression in NLC may be able to influence CLL differentiation and survival, a  
84 critical effect with implications in the design of novel anti-leukemic therapies.

85

86 **Conflict-of-interest disclosure:**

87 The authors declare no competing financial interests.

88

89 **Acknowledgments:**

90 This work was supported by grants from the National Council of Scientific and Technical  
91 Research (CONICET; Argentina), National Agency for Promotion of Science and  
92 Technology (Argentina), University of Buenos Aires and Fundación Sales (Argentina).

93

94 **Authors and affiliations:**

95 Diego O. Croci<sup>1</sup>, Pablo E. Morande<sup>2</sup>, Sebastián Dergan Dylon<sup>1</sup>, Mercedes Borge<sup>2</sup>, Marta A.  
96 Toscano<sup>1</sup>, Juan C. Stupirski<sup>1</sup>, Raimundo F. Bezares<sup>3</sup>, Julio Sánchez Avalos<sup>4</sup>, Marina  
97 Narbaitz<sup>2</sup>, Romina Gamberale<sup>2</sup>, Gabriel A. Rabinovich<sup>1\*</sup>, Mirta Giordano<sup>2\*</sup>

98

99 <sup>1</sup>Laboratorio de Inmunopatología, Instituto de Biología y Medicina Experimental (IBYME),  
100 Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), <sup>2</sup>Laboratorio de  
101 Inmunología Oncológica, Instituto de Investigaciones Médicas (IMEX/CONICET),  
102 Academia Nacional de Medicina, <sup>3</sup>Hospital General de Agudos ‘Dr. T. Alvarez’ and  
103 <sup>4</sup>Hospital de Clínicas ‘José de San Martín’, Ciudad de Buenos Aires, Argentina.

104

105 Corresponding author:

106 Dr Mirta Giordano, Laboratorio de Inmunología Oncológica, IMEX/CONICET, Academia

107 Nacional de Medicina, Pacheco de Melo 3081 (1425) Ciudad de Buenos Aires, Argentina.

108 Phone: 54 (11) 4805 34 11

109 Fax : 54 (11) 4803 95 73

110 E-mail: [mgjordano@hematologia.anm.edu.ar](mailto:mgjordano@hematologia.anm.edu.ar)

111

112

113 **References:**

114 1. Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. *N Engl J Med*  
115 2005; **352**: 804-815.

116

117 2. Riches JC, Ramsay AG, Gribben JG. T-cell function in chronic lymphocytic  
118 leukaemia. *Semin Cancer Biol* 2010; **20**: 431-438.

119

120 3. Abroun S, Otsuyama K, Shamsasenjan K, Islam A, Amin J, Iqbal MS *et al.*  
121 Galectin-1 supports the survival of CD45RA(-) primary myeloma cells in vitro. *Br J*  
122 *Haematol* 2008; **142**: 754-765.

123

124 4. Rabinovich GA, Croci DO. Regulatory circuits mediated by lectin-glycan  
125 interactions in autoimmunity and cancer. *Immunity* 2012; **36**: 322-335.

126

127 5. Juszczynski P, Ouyang J, Monti S, Rodig SJ, Takeyama K, Abramson J *et al.* The  
128 AP1-dependent secretion of galectin-1 by Reed Sternberg cells fosters immune  
129 privilege in classical Hodgkin lymphoma. *Proc Natl Acad Sci U S A* 2007; **104**:  
130 13134-13139.

- 131 6. Burger JA, Tsukada N, Burger M, Zvaifler NJ, Dell'Aquila M, Kipps TJ. Blood-  
132 derived nurse-like cells protect chronic lymphocytic leukemia B cells from  
133 spontaneous apoptosis through stromal cell-derived factor-1. *Blood* 2000; **96**: 2655-  
134 2663.
- 135
- 136 7. Morande PE, Zanetti SR, Borge M, Nannini P, Jancic C, Bezares RF *et al.* The  
137 cytotoxic activity of Aplidin in chronic lymphocytic leukemia (CLL) is mediated by  
138 a direct effect on leukemic cells and an indirect effect on monocyte-derived cells.  
139 *Invest New Drugs* 2012; **30**: 1830-1840.
- 140
- 141 8. Ilarregui JM, Croci DO, Bianco GA, Toscano MA, Salatino M, Vermeulen ME *et al.*  
142 Tolerogenic signals delivered by dendritic cells to T cells through a galectin-1-  
143 driven immunoregulatory circuit involving interleukin 27 and interleukin 10. *Nat*  
144 *Immunol* 2009; **10**: 981-991.
- 145
- 146 9. Fayad L, Keating MJ, Reuben JM, O'Brien S, Lee BN, Lerner S *et al.* Interleukin-6  
147 and interleukin-10 levels in chronic lymphocytic leukemia: correlation with  
148 phenotypic characteristics and outcome. *Blood* 2001; **97**: 256-263.
- 149
- 150 10. Sivina M, Hartmann E, Kipps TJ, Rassenti L, Krupnik D, Lerner S *et al.* CCL3  
151 (MIP-1alpha) plasma levels and the risk for disease progression in chronic  
152 lymphocytic leukemia. *Blood* 2011; **117**: 1662-1669.
- 153
- 154 11. Yan XJ, Dozmorov I, Li W, Yancopoulos S, Sison C, Centola M *et al.* Identification  
155 of outcome-correlated cytokine clusters in chronic lymphocytic leukemia. *Blood*  
156 2011; **118**: 5201-5210.

- 157 12. Packham G, Stevenson F. The role of the B-cell receptor in the pathogenesis of  
158 chronic lymphocytic leukaemia. *Semin Cancer Biol* 2010; **20**: 391-399.  
159
- 160 13. Gauthier L, Rossi B, Roux F, Termine E, Schiff C. Galectin-1 is a stromal cell  
161 ligand of the pre-B cell receptor (BCR) implicated in synapse formation between  
162 pre-B and stromal cells and in pre-BCR triggering. *Proc Natl Acad Sci U S A* 2002;  
163 **99**: 13014-13019.  
164
- 165 14. Yang M, Hase H, Legarda-Addison D, Varughese L, Seed B, Ting AT. B cell  
166 maturation antigen, the receptor for a proliferation-inducing ligand and B cell-  
167 activating factor of the TNF family, induces antigen presentation in B cells. *J*  
168 *Immunol* 2005; **175**: 2814-2824.  
169
- 170 15. Zucchetto A, Benedetti D, Tripodo C, Bomben R, Dal Bo M, Marconi D *et al.*  
171 CD38/CD31, the CCL3 and CCL4 chemokines, and CD49d/vascular cell adhesion  
172 molecule-1 are interchained by sequential events sustaining chronic lymphocytic  
173 leukemia cell survival. *Cancer Res* 2009; **69**: 4001-4009.  
174  
175  
176  
177  
178



179 **Legends to Figures:**

180

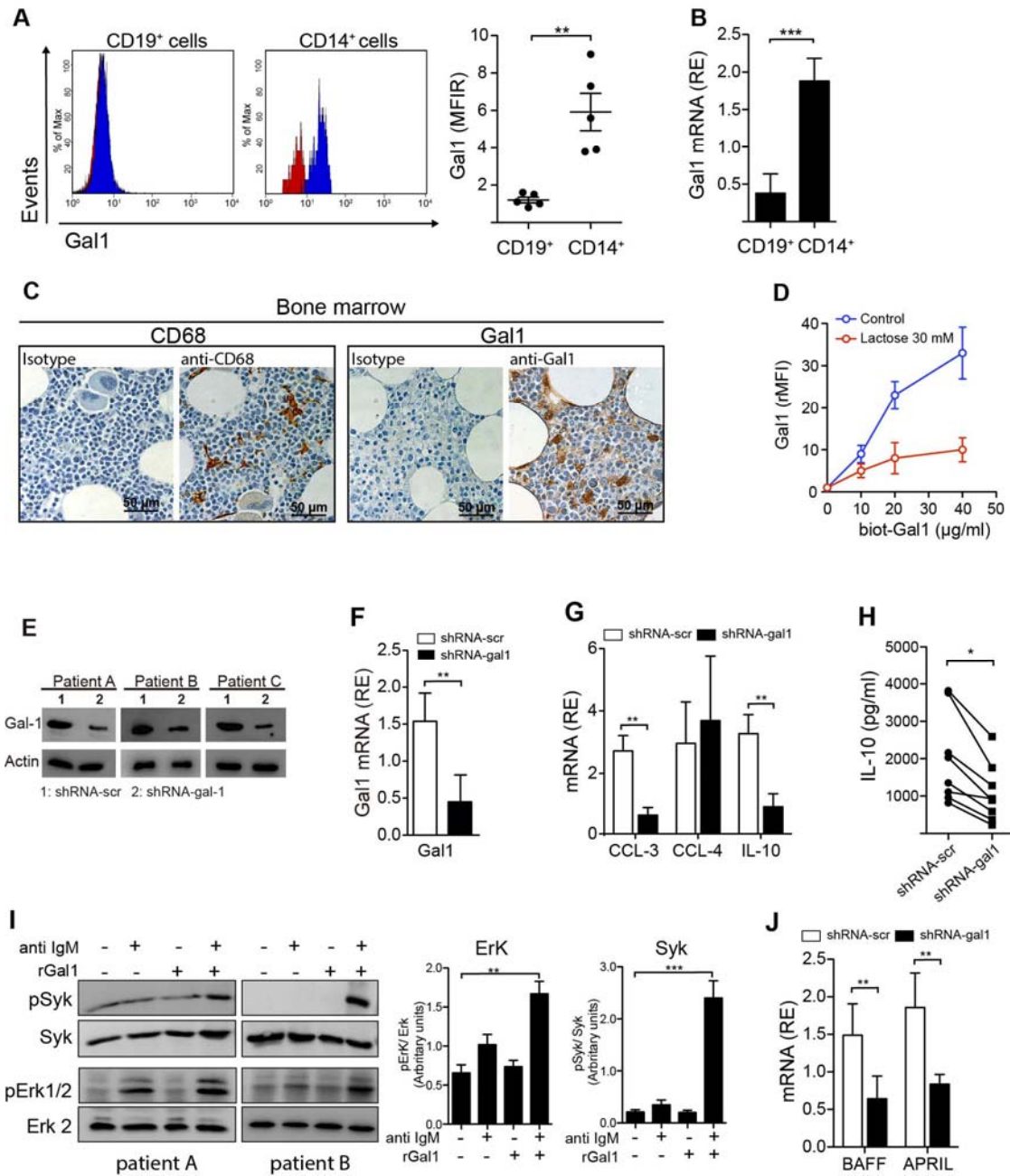
181 **Figure 1:** Myeloid cell-derived Gal1 modulates CLL cell function and signaling. (A-D)  
182 Gal1 expression in peripheral blood and bone marrow from CLL patients and binding of  
183 Gal1 to leukemic B cells. (A) Gal1 expression was assessed in fixed and permeabilized  
184 PBMC from CLL patients by flow cytometry. B lymphocytes (> 98% CLL cells) and  
185 monocytes were discriminated by CD19 and CD14 expression. Results are shown as mean  
186 fluorescence intensity ratio (MFIR) (n=5). Left, representative histograms (Red: isotype  
187 control, Blue: Gal1) in CD19- or CD14-expressing cells. \*\* p<0.01. (B) mRNA levels of  
188 Gal1 in CD19<sup>+</sup> or CD14<sup>+</sup> cells from CLL patients. Data are the mean ± SD (n=9) \*\*\*  
189 p<0.001. RE: band intensity relative to actin. (C) Immunoperoxidase staining of Gal1 in  
190 CLL bone marrow aspirates. Strong Gal1 expression was detected in a minor fraction of  
191 cells associated with infiltrating lymphocytes, which correspond to CD68<sup>+</sup> cells showing  
192 macrophage or dendritic cell-like morphology. (D) PBMC from CLL patients were  
193 incubated with increasing concentrations of biotin-conjugated Gal1 in the presence or  
194 absence of lactose 10 mM and washed before tagging with streptavidin-FITC. Viable  
195 leukemic cells were discriminated by forward-scatter gating and CD19 labeling and  
196 analyzed by flow cytometry. Results are shown as rMFI of Gal1 binding. Values are the  
197 mean ± SD from 9 CLL samples evaluated. rMFI (relative mean fluorescence intensity) =  
198 (MFI with Gal1 – MFI without Gal1) / MFI without Gal1. (E-I) Inhibition of Gal1  
199 expression in NLC affects leukemic B cell responses. *In vitro* differentiated NLC from CLL  
200 patients were infected with Gal1-specific shRNA (shRNA-Gal1) or scrambled control  
201 shRNA (shRNA-scr) and analyzed thereafter for Gal1 expression. (E) Western blot analysis  
202 and (F) mRNA levels of Gal1 in NLC following siRNA silencing (n=9), (\*\* p<0.01;  
203 Student's t-test). (G) mRNA expression of CCL3, CCL4 and IL-10 in CLL cells incubated  
204 for 72 h with transduced NLC. Results are the mean ± SD; n=9. (G) Secretion of IL-10 from

205 in CLL cells incubated for 72 h with transduced NLC. Results are the mean SD; n=8. (I)  
206 Analysis of BCR signaling in Gal1-treated CLL cells. Immunoblot of Syk and Erk1/2  
207 phosphorylation in cells incubated for 5 min with anti-IgM (0.1  $\mu$ M), Gal1 (3  $\mu$ M) or Gal1 +  
208 anti-IgM. Two representative out of seven experiments corresponding each to an individual  
209 patient are shown (left). (J) mRNA expression of BAFF and APRIL in transduced NLC at  
210 72 h of cell culture. Results are the mean  $\pm$  SD; n=9. \*\* p<0.01.

211

212 **Figure 2:** Expression of Gal1 in plasma and bone marrow samples from CLL patients. (A)  
213 Plasma levels of Gal1 in age-matched healthy donors and CLL patients. p<0.0001. (B)  
214 Plasma levels of Gal1 in CD38<sup>-</sup> ZAP70<sup>-</sup> (low risk) and CD38<sup>+</sup> and/or ZAP70<sup>+</sup> (high risk)  
215 CLL patients. (C-E) Gal1 expression in bone marrow samples from 6 stable and 7  
216 progressive CLL patients. (C) Semiquantitative analysis of Gal1 expression within CLL-  
217 infiltrating areas from stable or progressive patients. n=13 p=0.012 (Mann Whitney *U* test).  
218 (D) Analysis of presence of CD68<sup>+</sup> cells in CLL-infiltrating areas. n=11 p=0.008 (Mann  
219 Whitney *U* test). (E) Representative images from stable or progressive CLL biopsies  
220 showing Gal1 (left) or CD68 (Right) expression are shown.

FIGURE 1

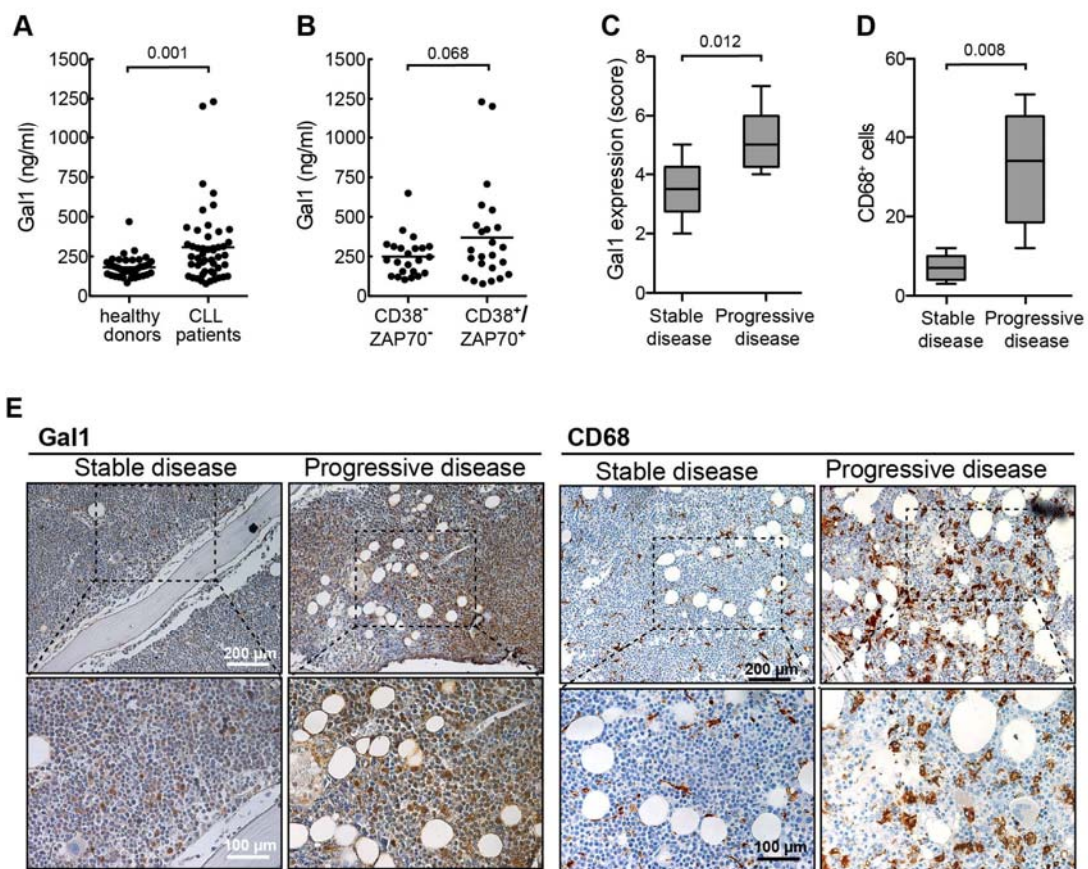


221

222

223

FIGURE 2



224

Accepted