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

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Letter to the Editor

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

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

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

Since B-cell receptor (BCR) signaling plays a central role in the survival, proliferation and trafficking of CLL cells[12], we evaluated whether Gal1 can modulate this

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

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

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

Conflict-of-interest disclosure:

The authors declare no competing financial interests.

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Authors and affiliations:

Diego O. Croci[1], Pablo E. Morande[2], Sebastián Dergan Dylon[1], Mercedes Borge[2], Marta A. Toscano[1], Juan C. Stupirski[1], Raimundo F. Bezares[3], Julio Sánchez Avalos[4], Marina Narbaitz[2], Romina Gamberale[2], Gabriel A. Rabinovich[1*], Mirta Giordano[2 *]

[1]Laboratorio de Inmunopatología, Instituto de Biología y Medicina Experimental (IBYME), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), [2]Laboratorio de Inmunología Oncológica, Instituto de Investigaciones Médicas (IMEX/CONICET), Academia Nacional de Medicina, [3]Hospital General de Agudos 'Dr. T. Alvarez' and [4]Hospital de Clínicas 'José de San Martín', Ciudad de Buenos Aires, Argentina.

Corresponding author:

Dr Mirta Giordano, Laboratorio de Inmunología Oncológica, IMEX/CONICET, Academia Nacional de Medicina, Pacheco de Melo 3081 (1425) Ciudad de Buenos Aires, Argentina.

Phone: 54 (11) 4805 34 11

Fax : 54 (11) 4803 95 73

E-mail: mgiordano@hematologia.anm.edu.ar

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Legends to Figures:

Figure 1: Myeloid cell-derived Gal1 modulates CLL cell function and signaling. (A-D) Gal1 expression in peripheral blood and bone marrow from CLL patients and binding of Gal1 to leukemic B cells. (A) Gal1 expression was assessed in fixed and permeabilized PBMC from CLL patients by flow cytometry. B lymphocytes (> 98% CLL cells) and monocytes were discriminated by CD19 and CD14 expression. Results are shown as mean fluorescence intensity ratio (MFIR) (n=5). Left, representative histograms (Red: isotype control, Blue: Gal1) in CD19- or CD14-expressing cells. ** $p<0.01$. (B) mRNA levels of Gal1 in $CD19^+$ or $CD14^+$ cells from CLL patients. Data are the mean ± SD (n=9) *** $p<0.001$. RE: band intensity relative to actin. (C) Immunoperoxidase staining of Gal1 in CLL bone marrow aspirates. Strong Gal1 expression was detected in a minor fraction of cells associated with infiltrating lymphocytes, which correspond to $CD68^+$ cells showing macrophage or dendritic cell-like morphology. (D) PBMC from CLL patients were incubated with increasing concentrations of biotin-conjugated Gal1 in the presence or absence of lactose 10 mM and washed before tagging with streptavidin-FITC. Viable leukemic cells were discriminated by forward-scatter gating and CD19 labeling and analyzed by flow cytometry. Results are shown as rMFI of Gal1 binding. Values are the mean ± SD from 9 CLL samples evaluated. rMFI (relative mean fluorescence intensity) = (MFI with Gal1 – MFI without Gal1) / MFI without Gal1. (E-I) Inhibition of Gal1 expression in NLC affects leukemic B cell responses. *In vitro* differentiated NLC from CLL patients were infected with Gal1-specific shRNA (shRNA-Gal1) or scrambled control shRNA (shRNA-scr) and analyzed thereafter for Gal1 expression. (E) Western blot analysis and (F) mRNA levels of Gal1 in NLC following siRNA silencing (n=9), (** $p<0.01$; Student´s t-test). (G) mRNA expression of CCL3, CCL4 and IL-10 in CLL cells incubated for 72 h with transduced NLC. Results are the mean ± SD; n=9. (G) Secretion of IL-10 from

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

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

FIGURE 1

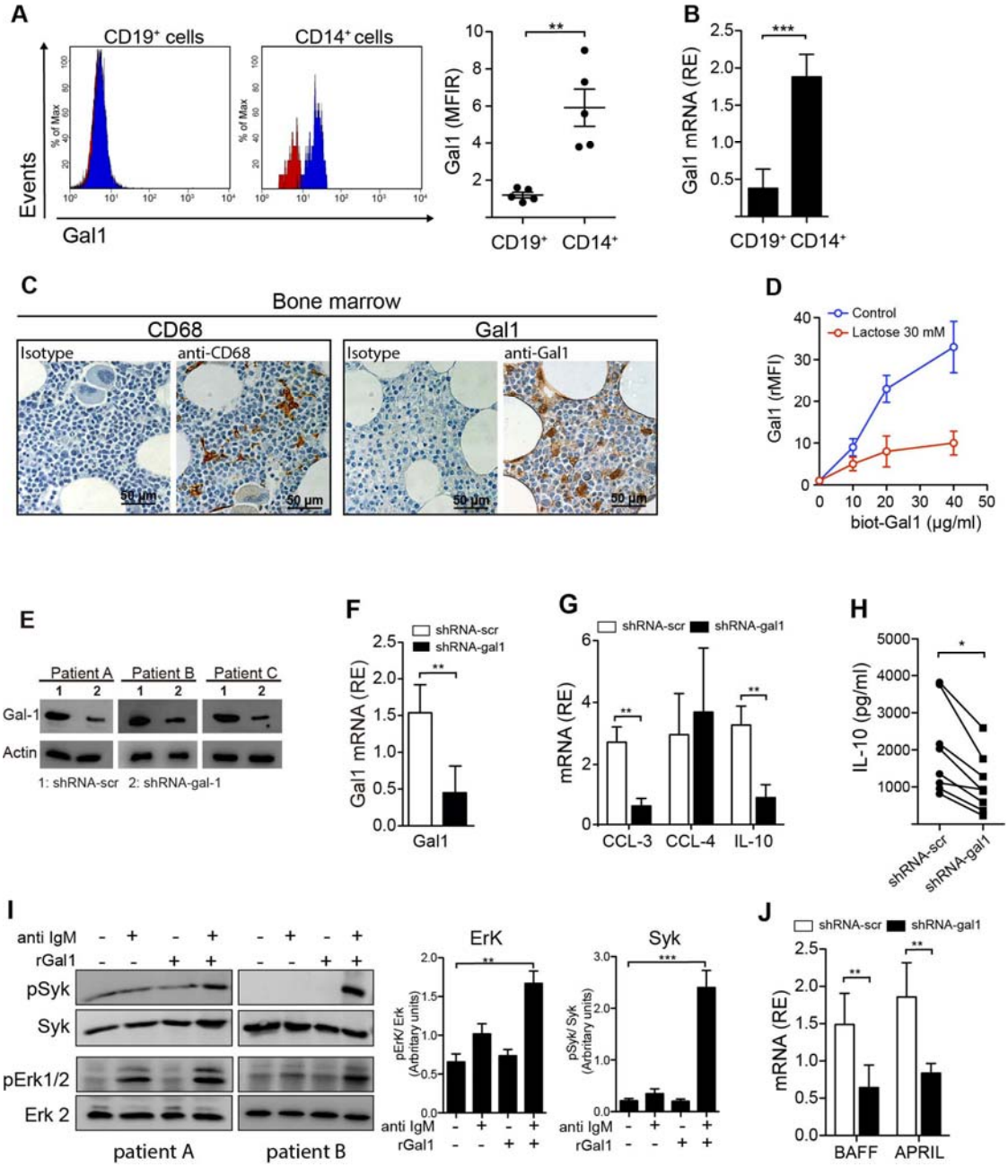
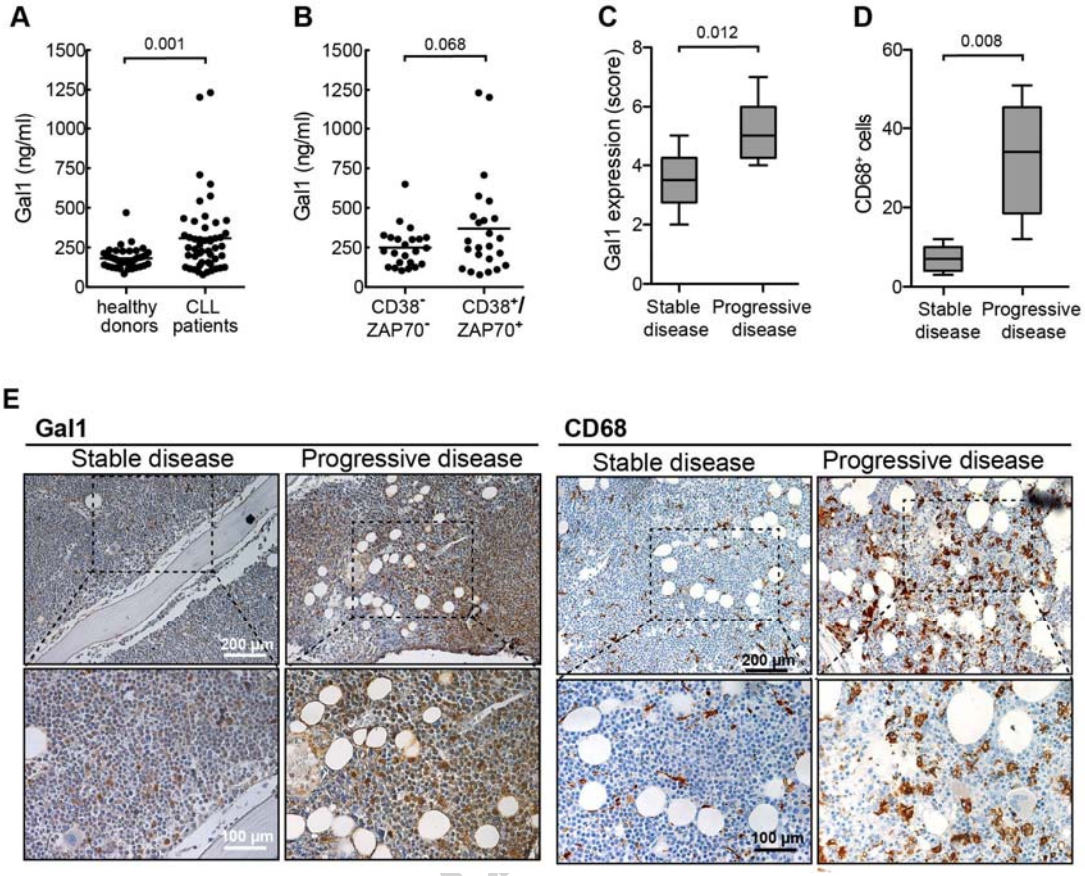


FIGURE 2



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