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FcγRIIb expression in early stage Chronic Lymphocytic Leukemia

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Abstract

In normal B-cells, B-cell antigen receptor (BCR) signaling can be negatively regulated by the low-affinity receptor FcγRIIb (CD32b). To better understand the role of FcγRIIb in chronic lymphocytic leukemia (CLL), we correlated its expression on 155 samples from newly-diagnosed Binet A patients with clinical characteristics and outcome. FcγRIIb expression was similar in normal B-cells and leukemic cells, this being heterogeneous among patients and within CLL clones. FcγRIIb expression did not correlate with well known prognostic markers [disease stage, serum beta-2 microglobulin (B2M), *IGHV* mutational status, expression of ZAP-70 and CD38, and cytogenetics] except for a weak concordance with CD49d. Moreover, patients with low

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Fc γ RIIb expression (69/155, 44.5%) required therapy earlier than those with high Fc γ RIIb expression (86/155, 55.5%) (median 151.4 months vs. not reached; $p=0.071$). These results encourage further investigation on the role of Fc γ RIIb in CLL biology and prognostic significance in larger series of patients.

Keywords

Chronic lymphocytic leukemia; Fc γ RIIb; B-cell receptor; treatment-free survival; B-cell activation

Introduction

Chronic lymphocytic leukemia (CLL), the most common adult B-cell malignancy in Western countries, is characterized by the accumulation of monoclonal CD5+ B-cells in peripheral blood, bone marrow, and lymphoid tissues [1]. The clinical course of CLL is extremely heterogeneous. While in some patients the disease can remain stable for years, others present with active disease and short survival [2]. This clinical diversity is a reflection of the biologic heterogeneity of the disease [3–5], in which the B-cell antigen receptor (BCR) plays a crucial role [6]. Patients with clones bearing mutated immunoglobulin heavy chain variable (*IGHV*) genes have significantly superior survival compared with patients with unmutated *IGHVs* [7,8].

Signaling through the BCR on B-cells can be regulated by the inhibitory receptor Fc γ RIIb [9,10]. Fc γ RIIb belongs to the low affinity Fc γ RII (CD32) family, which comprises three isoforms with highly homologous structure but opposing functions. While Fc γ RIIa and Fc γ RIIc are activating receptors, Fc γ RIIb mediates inhibitory signals [11]. In particular, Fc γ RIIb exerts a negative effect when colligated with the BCR by cognate immune complexes [12] helping to maintain peripheral tolerance and shape the B-cell repertoire [13,14].

The role of Fc γ RIIb as a negative regulator of BCR signaling has been extensively studied in normal B-cells [15–21] and in human autoimmune diseases [13,22,23]. However, less is known about its expression and signaling on leukemic cells [24–26]. These studies used pan-specific monoclonal antibodies (mAbs) which do not discriminate between the Fc γ RIIa and Fc γ RIIb isoforms and reported discordant results.

Moreover, there is some indication that Fc γ RIIb-BCR colligation may inhibit BCR signaling in CLL cells, thus reproducing its role in normal B-cells [27]. Considering the activated nature of CLL cells [28,29] and the major role of BCR in the pathogenesis of the disease [6], analyzing the expression of Fc γ RIIb in this malignancy might provide information not only about the mechanisms that regulate leukemic cell activation but also about the clinical behavior of the disease.

The aims of the present study were to: (1) analyze the baseline expression of Fc γ RIIb on CLL cells and normal B-cells by flow cytometry using an Alexa Fluor 488-conjugated mAb specific for the human isoform of Fc γ RIIb and (2) explore the relationship between Fc γ RIIb expression levels and well known prognostic markers in this disease.

Methods

Study population

The study population included 149 patients with Binet A CLL and 6 patients with monoclonal B-cell lymphocytosis (MBL), seen at two university hospitals in Barcelona (Hospital Clinic and Hospital de la Santa Creu i Sant Pau, Barcelona, Spain). The selection of the patients was based on the availability of cryopreserved peripheral blood samples before treatment. Patients were diagnosed according to the criteria of the International Workshop on CLL [30]. The main clinical and biological characteristics of patients at diagnosis are summarized in Table SI. Peripheral blood samples from 34 healthy blood donors were obtained for comparative purposes from the Blood and Tissue Bank of Catalonia. Study approval was obtained from the Research Ethics Committees in agreement with the declaration of Helsinki.

Flow cytometry

FcγRIIb expression was assessed on peripheral blood mononuclear cells (PBMCs) from patients with CLL and healthy individuals using a specific Alexa Fluor 488-conjugated chimeric IgG anti-human FcγRIIb mAb (clone number: ch2B6N297Q) [31,32], which was kindly provided by MacroGenics (Rockville, MD). We used the following four-colour flow cytometry combinations: FcγRIIb/CD38/CD19/CD5 and FcγRIIb/CD49d/CD19/CD5. Briefly, PBMCs were suspended at a concentration of 10^7 cells per milliliter in fluorescent-activated cell sorter (FACS) buffer. Antibodies were added to 100μl of cell suspension, incubated for 30 minutes at 4°C, and washed with FACS buffer (1% BSA and 0.1% sodium azide in PBS) before analysis on a flow cytometer (FACS CantoII, Becton Dickinson, San Jose, CA). Data were analyzed using FlowJo v10 software. Isotype controls were run for each sample. Results were expressed as the mean fluorescence intensity ratio (MFIR) between the MFI for FcγRIIb and the MFI for the isotype of the corresponding control mAb.

We assessed FcγRIIb expression levels on paired samples collected at different time points after diagnosis and before treatment from 10 patients with CLL. The median interval sampling was 10.6 months (4.8–19.7). We confirmed that FcγRIIb expression remained stable over time in non-treated patients (Figure S1).

Determination of biological prognostic factors

Fluorescent in-situ hybridization (FISH) studies for 11q, 13q and 17p deletions and trisomy 12, as well as *IGHV-IGHD-IGHJ* rearrangements and mutational status were assessed as previously described [33]. CD38, ZAP-70 and CD49d expression were evaluated by multiparameter flow cytometry using a BD FACS Canto II cytometer. A cut-off of 30% was used for CD38 and CD49d [7,34], and 20% was used for ZAP-70 [35].

Statistical analysis

Comparison between groups was performed using the Fisher's exact test for categorical variables and Mann-Whitney U test for continuous variables. Treatment-free survival (TFS) was defined as the interval between the date of diagnosis and the date of first treatment,

death, or last follow-up. Those patients with a TFS superior or equal to 15 years (180 months) were censored at 180 months, since very few patients were at risk beyond this time point (only 10% of the study cohort). We used ROC curves to determine the cut-off of Fc γ RIIb MFIR that could better discriminate two subgroups with different TFS probability, this being 45.4. Survival curves were obtained using the Kaplan–Meier method, and the differences between the subgroups were compared using the log-rank test. Two-sided *p*-values of <0.05 were considered to be significant. Statistical calculations were performed with SPSS software (version 22.0; SPSS, Chicago, IL, USA).

Results

Expression of Fc γ RIIb on CLL and normal B-cells

The surface membrane expression of Fc γ RIIb was analyzed in 155 samples from patients with Binet A CLL and 34 from healthy donors. All CD5+CD19+ leukemic cells from patients with CLL and all CD19+ normal B-cells from healthy donors expressed Fc γ RIIb (Figure 1A). The median MFIR for Fc γ RIIb on CLL cells (median MFIR: 47.6, range: 8.3–153.4) was similar to that observed on CD19+ normal B-cells (median MFIR: 49.4, range: 30.8–73.2) (Figure 1B). However, the MFIR for Fc γ RIIb on leukemic cells was heterogeneous, as shown in Figure 1B, being twofold higher than the median MFIR levels in 10 out of 155 (6.5%) samples.

We further observed that within individual CLL clones, the CD49d+ fractions had higher expression levels of Fc γ RIIb than CD49– cells [median: 60.5 (9.8–115.4) vs. 43.2 (7.0–103.0), *p*<0.001]. In addition, the expression of Fc γ RIIb was higher on CD38+ compared to CD38– cells [median MFIR: 52.6 (11.3–125.5) vs. 45.5 (8.0–159.2), *p*=0.010] (Figure 1C).

Expression of Fc γ RIIb and association with other prognostic markers

Eighty-six out of 155 patients (55.5%) showed high Fc γ RIIb expression (MFIR $\geq$ 45.4) and 69 (44.5%) showed low Fc γ RIIb expression (MFIR<45.4). The main characteristics according to Fc γ RIIb expression are shown in Table I. We did not find any association between Fc γ RIIb expression levels and well known prognostic markers [disease stage, serum beta-2 microglobulin (B2M), *IGHV* mutational status, expression of ZAP-70 and CD38, and FISH cytogenetics], except for age and CD49d expression. The concordance between Fc γ RIIb and CD49d expression was statistically significant but weak (κ =0.185, *p*=0.004), also when analyzed as continuous variables (Spearman's rho=0.306, *p*<0.001).

Correlation of Fc γ RIIb and disease outcome

After a median follow up of 6.5 years (0.2–15.0), 33/155 patients (21%) required therapy and 13/155 (8%) died. There were no differences in treatment modalities between patients with low and high Fc γ RIIb expression levels, and no significant differences in response rate were observed [39% complete response (CR) vs. 20% CR, *p*= 0.283]. In patients with low Fc γ RIIb expression there was a trend for a shorter TFS than those with high Fc γ RIIb expression (median 151.4 months vs. not reached; log-rank *p*=0.071) (Figure 2). Other prognostic parameters including B2M, LDH, expression of ZAP70, CD38 and CD49d, *IGHV* mutational status, and FISH cytogenetics significantly correlated with shorter TFS by

univariate analysis (Table II). Multivariate analysis was not performed due to the small sample size.

Discussion

CLL is a disease of partially activated B-lymphocytes [28,29]. However, the degree that ongoing suppression mediated by inhibitory receptors plays in this abortive activation is unknown. The inhibitory receptor Fc γ RIIb regulates the activation of normal B-cells by colligation with the BCR [21], and there is evidence of a role for a defective Fc γ RIIb inhibition in autoimmune diseases [13,22,23] to which incidentally patients with CLL are predisposed [36]. At the best of our knowledge, there is only one study suggesting that in CLL Fc γ RIIb plays a role in controlling BCR activation in CLL [27]. In the present work, we analyzed the expression levels of Fc γ RIIb on CD19⁺ normal B-cells and CD5⁺CD19⁺ CLL cells by using a specific antibody against human Fc γ RIIb we found that the median levels of Fc γ RIIb were similar in both normal B-cells and CLL cells. Although a number of studies have investigated the expression of Fc γ RII on both human normal B-cells and CLL [24–26], none of them used an antibody specific for its inhibitory isoform Fc γ RIIb. In this regard, Fc γ RIIb is the only Fc receptor expressed on murine B-cells [37], but human normal B-cells and CLL cells can also express the activating Fc γ RIIa isoform [27,38], underlying the importance of using specific antibodies against human Fc γ RIIb. In addition, while one study reported a decreased expression of Fc γ RIIb on CLL cells [26], others described that Fc γ RIIb expression levels were similar in CLL cells and normal B-lymphocytes [24,25]. These latter contributions are in agreement with our observations, although in our study the expression of Fc γ RIIb on CLL cells was heterogeneous, not only among patients but also within cell clones.

CD49⁺ or CD38⁺ cells showed higher Fc γ RIIb expression levels than CD49[–] or CD38[–] cells, an observation in keeping with the intraclonal heterogeneity of CLL [39,40] and relevant as CD38⁺ and CD49d⁺ CLL cells are more responsive to BCR ligation than their negative counterparts [41–44]. Therefore, the upregulation of Fc γ RIIb in CD49d⁺ and CD38⁺ compartments may reflect an attempt to modulate the activation mediated by BCR in these clones, a hypothesis that deserves further investigation.

Although the potential negative regulatory signaling mediated by the colligation of Fc γ RIIb-BCR in CLL cells has been scarcely studied, there is some indication that this colligation may be functional in CLL through the inhibition of BCR-induced ERK phosphorylation [27]. BCR signaling is involved in survival, proliferation and migration of CLL cells [6,45] and Fc γ RIIb is capable of inhibiting this signaling, which is in agreement with the correlation between high Fc γ RIIb expression levels and a longer TFS as found in our study. Although based in a small number of cases, we did not find any meaningful association between the expression of Fc γ RIIb and other prognostic factors including *IGHV* mutational status, cytogenetics and expression of ZAP-70, CD38 or CD49d.

In conclusion, we have shown that median expression levels of Fc γ RIIb were similar between healthy individuals and CLL patients. However, in the latter group the expression of Fc γ RIIb is heterogenous and variable within CLL subclones. Furthermore, a trend for a

longer TFS was observed in patients with higher Fc γ RIIb levels in agreement with the role of this receptor as a negative regulator of BCR. Further studies to deepen our understanding on the pathophysiological role of Fc γ RIIb in CLL as well as its potential prognostic significance are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. FcγRIIb expression levels on normal human B-cells and CLL cells

(A) Representative histograms of FcγRIIb expression (white histograms). Dark gray and light gray histograms indicate isotype and unstained controls, respectively. Expression levels of FcγRIIb (B) on CLL cells from patients (n=155) and normal B-cells from healthy donors (n=34); and (C and D) within clones of the same patients. The thick horizontal lines represent the median values, and the lower and upper boundaries of the boxes the interquartile ranges. Bars encompass the data points within 1.5 times the interquartile range from the first or third quartile. Filled circles represent outliers. FcγRIIb expression is expressed as the mean fluorescence intensity ratio (MFIR) between the MFI for FcγRIIb and the MFI for the corresponding isotype. *p*-values were calculated using the U-Mann Whitney test.

Figure 2. Kaplan-Meier curves for treatment free survival (TFS) in patients with CLL based on FcγRIIb expression levels

The median TFS for patients with high FcγRIIb expression was not reached compared with 151.4 months for patients with low FcγRIIb expression. *p*-values were calculated using the Log-rank test.

Table I

Clinical and biological characteristics of patients with CLL according to the expression of FcγRIIb

	FcγRIIb expression		p-value
	Low (n=69)	High (n=86)	
Median age (years)	67.0 (40.3–88.6)	70.4 (46.7–88.2)	0.057
Median follow-up (years)	7.3 (0–15.0)	6.1 (0.1–15.0)	0.740
Gender			
Female (n= 72)	32 (20)	40 (26)	1.000
Male (n= 83)	37 (24)	46 (30)	
Median lymphocyte count (×10⁹/l)	10.4 (5.2–87.3)	11.1 (5.1–73.2)	0.254
Median Hb (g/l)	140 (87–167)	142 (105–171)	0.165
LDH elevated (n, %)	5/67 (7)	5/79 (6)	1.000
B2M elevated (n, %)	22/59 (41)	21/66 (32)	0.574
IGHV mutational status			
Mutated (n= 84)	37 (29)	47 (37)	0.578
Unmutated (n= 44)	17 (13)	27 (21)	
FISH cytogenetics			
Low risk (n= 113)	49 (39)	64 (50)	0.776
High risk* (n= 14)	5 (4)	9 (7)	
CD38			
<30% (n= 111)	52 (35)	59 (40)	0.445
30% (n= 36)	14 (10)	22 (15)	
CD49d			
<30% (n= 120)	61 (39)	59 (38)	0.004
30% (n= 35)	8 (5)	27 (18)	
ZAP-70			
<20% (n= 88)	37 (29)	51 (39.5)	0.568
20% (n= 41)	20 (15.5)	21 (16)	

Hb, hemoglobin; LDH, lactatedeshydrogenase; B2M, beta-2 microglobulin.

* High risk cytogenetics includes deletions 11q and/or 17p

Table II

Univariate COX regression analyses for TFS.

	HR (95%CI)	<i>p</i>
FcγRIIb (low vs. high)	1.7 (1.0–3.0)	0.074
Age (≥ 65 vs. <65)	1.3 (0.7–2.4)	0.357
FISH cytogenetics (high risk vs. low risk)	4.2 (1.9–9.5)	<0.001
IGHV mutational status (unmutated vs. mutated)	7.3 (3.8–14.1)	<0.001
LDH (elevated vs. normal)	14.3 (5.8–35.5)	<0.001
B2M (elevated vs. normal)	2.9 (1.5–5.5)	0.001
CD38 (≥ 30% vs. <30%)	6.8 (3.6–12.6)	<0.001
CD49d (<30% vs. ≥ 30%)	2.2 (1.2–4.2)	0.016
ZAP-70 (<20% vs. ≥ 20%)	2.3 (1.2–4.3)	0.011

Hb, hemoglobin; LDH, lactatedeshydrogenase; B2M, beta-2 microglobulin.

* High risk cytogenetics includes deletions 11q and/or 17p