Leukemia & Lymphoma, 2015; Early Online: 1–8

ISSN: 1042-8194 print / 1029-2403 online
DOI: 10.3109/10428194.2014.957205

informa
healthcare

ORIGINAL ARTICLE: RESEARCH

Surface localization of high-mobility group nucleosome-binding protein 2 on leukemic B cells from patients with chronic lymphocytic leukemia is related to secondary autoimmune hemolytic anemia

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Abstract

Chronic lymphocytic leukemia (CLL) is the main cause of autoimmune hemolytic anemia (AHA). However, the cellular basis underlying this strong association remains unclear. We previously demonstrated that leukemic B cells from patients with CLL recognize the erythrocyte protein Band 3, a prevalent autoantigen in AHA. Here we show that the major binding site of Band 3 on leukemic cells is an extrinsic protein identified as high-mobility group nucleosome binding protein 2 (HMGN2), a nucleosome-interacting factor which has not been previously reported at the cell surface. T lymphocytes do not express HMGN2 or bind Band 3. Removal of HMGN2 from the cell membrane abrogated the capacity of Band 3-pulsed CLL cells to induce CD4 + T cell proliferation. We conclude that surface HMGN2 in leukemic B cells is involved in Band 3 binding, uptake and presentation to CD4 + T lymphocytes, and as such may favor the initiation of AHA secondary to CLL.

Keywords: Autoimmune hemolytic anemia, chronic lymphocytic leukemia, band 3, HMGN2, B cells

Introduction

Autoimmune complications are common in patients with chronic lymphocytic leukemia (CLL), among which autoimmune hemolytic anemia (AHA) is the most frequent manifestation [1–3]. In this process, autoantibodies targeting surface membrane proteins lead erythrocytes to accelerated destruction by splenic and hepatic macrophages. Although the association between AHA and CLL is well established, the pathogenesis remains largely unknown. In most leukemic patients, AHA is due to warm reactive polyclonal immunoglobulin G (IgG) antibodies that are not produced by the malignant clone, but by residual normal B cells [1,3]. While it is clear that CLL cells are not the source of pathogenic antibodies, they might be involved in the initiation of AHA by acting as aberrant antigen presenting cells. We tested this hypothesis and found that CLL cells are able to bind and internalize Band 3, the most abundant glycoprotein in the erythrocyte membrane and a prevalent autoantigen in AHA [4]. If appropriately stimulated, Band 3-pulsed CLL cells induce CD4 + T-cell proliferation by a human leukocyte antigen (HLA)-DR-dependent mechanism, indicating that antigen presentation is involved [4].

Band 3, also termed anion exchanger 1 (AE1), is a predominant membrane transporter in red blood cells and plays a central role in their structure and function [5,6]. It can be cleaved into two independent structural domains. The 55 kDa membrane-spanning domain serves to catalyze the exchange of anions across the membrane and also mediates removal of senescent erythrocytes from circulation [5–7]. The N-terminal cytoplasmic domain of Band 3 functions primarily as an anchoring site for other membrane-associated proteins, including ankyrin, protein 4.2 and aldolase, among others [5,8]. Band 3 also contains a single complex N-linked oligosaccharide that is attached to asparagine in the fourth extracellular loop [7].

There are two well-characterized receptors for Band 3 on human cells: the scavenger receptor CD36 and the multifunctional shuttling protein nucleolin [9,10]. CD36 is responsible for the adherence of altered Band 3 to monocytes and

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Received 2 June 2014; revised 7 June 2014; accepted 7 August 2014

endothelial cells in the case of plasmodium-infected erythrocytes. On the other hand, nucleolin binds the conformational motif of aggregated Band 3 glycan that is induced by oxidation in senescent erythrocytes. Although at least some clones of CLL cells express CD36 and nucleolin [11,12], we found that none of these receptors were involved in the binding and endocytosis of Band 3 by leukemic B cells [4]. Indeed, by employing a recombinant N-terminal region of Band 3 (B3N), we showed that this was the relevant domain in the phenomenon we described and, more importantly, that erythrocyte vesicles that exposed B3N were specifically recognized by CLL cells [4]. Finally, and taking into account that many CLL clones express polyreactive B-cell receptors (BCRs) and that antigen processing for major histocompatibility complex (MHC) class II presentation in B cells mostly depends on BCR endocytosis, we also evaluated the involvement of the BCR in Band 3 recognition, without positive results. The aim of the present work was therefore the identification of the binding site(s) for Band 3 in malignant B cells from patients with CLL.

Materials and methods

CLL patient samples

Blood samples from patients with CLL were obtained after written informed consent in accordance with the Declaration of Helsinki. These studies were approved by the institutional review board of the National Academy of Medicine in Buenos Aires. CLL was diagnosed according to standard clinical and laboratory criteria. At the time of the analysis, all patients were free from clinically relevant infectious complications and had negative Coombs tests. Samples from patients who were either untreated or had not received chemotherapy for at least 6 months before investigation were used.

Reagents, antibodies and cell lines

Ficoll–Hypaque solution (Lymphoprep; Nycomed Pharma, Oslo, Norway), RPMI 1640 medium and phosphate buffered saline (PBS) were purchased from HyClone Laboratories Inc. (Logan, UT). Fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Invitrogen Life Technologies (Grand Island, NY). Fluorescent-conjugated monoclonal antibodies directed to human CDs were purchased from BD Biosciences (San Jose, CA). Goat anti-human high-mobility group nucleosome binding protein 2 (HMGN2) antibody and isotype control were from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescent secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). The Daudi cell line was obtained from the American Type Culture Collection (ATCC).

Proteins

Vector pET21a containing the cytoplasmic B3N with C terminus His6 tag (kindly provided by Dr. Yu Ding) was cloned into BL21 (D3) pLys for its expression, as previously described [13]. In brief, after isopropyl-β-D-thiogalactopyranoside induction, cellular lysis was performed, and the supernatant obtained after centrifugation was first purified on a HisTrap HP-1 mL column (GE Healthcare) eluted with 0.5 M imidazole. Final purification was obtained on a Resource Q column (GE Healthcare). Purified protein was concentrated and stored at −80°C. Whole Band 3 protein was purified from pooled erythrocytes of healthy volunteers by a modification of the method of Casey *et al.* [14]. In brief, erythrocyte ghosts were stripped of peripheral proteins and solubilized in deoxycholate 1% m/v in low ionic strength buffer to selectively act on B3 and not the glycophorins [15]. Excess detergent and contaminating proteins were removed by diethylaminoethyl (DEAE) anion exchange chromatography and dialysis. Finally, the protein was concentrated and stored at −80°C. Recombinant human HMGN2 protein was kindly given by Dr. James Kadonaga from the University of San Diego, CA.

Binding assays

Peripheral blood mononuclear cells (PBMCs) were isolated by density gravity centrifugation (Lymphoprep; Nycomed Pharma). Binding of B3N was assayed by fluorescence activated cell sorting (FACS). PBMCs (5×10^5 cells) were incubated on ice with different protein concentrations for 20 min in PBS with bovine serum albumin (BSA) 0.5% and then washed three times with the same buffer. Binding of B3N was revealed using a polyclonal mouse specific anti-serum, obtained with a standard immunization protocol followed by anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC). CLL cells were discriminated from non-leukemic lymphocytes using anti-CD19 and anti-CD5 fluorescent antibodies. Cells were acquired with a FACSCalibur flow cytometer (BD Biosciences) and analyzed with CellQuest software.

Removal of non-integral membrane proteins by acidic elution, B3N affinity chromatography and bound protein identification

To remove peripheral membrane proteins, 1×10^6 CLL cells were incubated in low pH buffer containing sodium citrate 0.05 M, potassium phosphate 0.1 M, pH 2.5 for 30 s at room temperature. Preparations were rapidly neutralized and washed three times in PBS prior to the binding assay. Recombinant B3N protein (11.6 mg) was coupled to AminoLink® agarose resin according to the manufacturer's procedure (Pierce; cat. no. 44894). Neutralized acidic elution preparations obtained from 100×10^6 CLL cells of three patients were independently loaded into the B3N column. After washing with PBS, bound proteins were eluted at NaCl 0.5 M. For their identification, a 4800 MALDI-TOF/TOF (matrix assisted laser desorption ionization time-of-flight/time-of-flight) instrument (Applied Biosystems) in positive ion reflector mode was employed, by using a matrix solution of α-cyano-4-hydroxycinnamic acid in 0.2% trifluoroacetic acid in acetonitrile–H_2O (50% v/v). External calibration was assessed using a mixture of standard peptides (Applied Biosystems).

ELISA

Reactivity of HMGN2 for B3N was confirmed using enzyme-linked immunosorbent assay (ELISA). HMGN2 or BSA (0.5 μg) was immobilized into each well of Costar EIA plates by 18 h incubation. Wells were blocked with 3% BSA in PBS

and increasing concentrations of B3N were added in triplicate. Plates were incubated for 2 h at room temperature, washed three times with PBS-Tween, and mouse anti-B3N serum was added for an additional 2 h. Interaction was revealed using a horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Jackson ImmunoResearch) and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate. The color was allowed to develop for 5 min, quenched with 1 M HCl, and the absorbance at 450 nm determined.

Confocal microscopy

PBMCs (3×10^6/mL) from patients with CLL were seeded on chambered coverglass slides (Nunc Lab-Tek; ThermoScientific, Waltham, MA). The images were acquired by sequentially scanning with settings optimal for Alexa488 fluorophore (488 nm excitation with argon laser line and detection of emitted light between 505 and 525 nm; pseudocoloured green), using a FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan) equipped with a Plapon 603/1.42 oil immersion objective.

Cell culture and assessment of cell proliferation

To evaluate the capacity of CLL cells to induce T cell proliferation upon Band 3 presentation, leukemic cells were prepared by negative selection with anti-CD2, -CD3, -CD14, -CD56 and -CD16 mouse IgG and magnetic beads coated with anti-mouse IgG (Pierce), according to the manufacturer's instructions. T cell-enriched fractions (T cells) were also prepared by negative selection with anti-CD14, -CD16, -CD56 and -CD19 CLL by the same procedure. T cell purity was $>95\%$ and CLL cell purity was always 99% or greater, assayed by FACS. All cultures were performed in RPMI 1640 supplemented with 10% autologous serum, 100 µg/mL streptomycin and 100 U/mL penicillin, in a humidified atmosphere of 5% CO_2/95% air. Purified CLL cells were seeded onto gamma-irradiated CD40L fibroblast monolayers for overnight stimulation. Then CLL cells were picked up, centrifuged and treated or not with buffer pH 2.5 to remove non-integral membrane proteins as indicated above. For antigen uptake, control and eluted CLL cells were incubated for 6 h with Band 3 (10 µg/mL) in new wells. CLL cells were then gently picked up, washed three times with culture medium to remove unbound antigen and gamma irradiated (30 Gy). Purified T cells from patients with CLL (10^7/mL) in PBS were incubated at 37°C for 10 min with 0.5 µM carboxyfluorescein succinimidyl ester (CFSE; Invitrogen). Staining was terminated by adding RPMI 1640 with 5% serum. Cells were thoroughly washed in PBS. Stained T cells and autologous activated CLL cells (pulsed with either Band 3 or medium alone, either previously treated or not with the acidic elution buffer) were co-cultured at 1:1 ratio at 10^6cells/mL for 5 days. Cells were harvested, washed in PBS plus 0.5% BSA, and stained on ice with anti-human CD4–peridinin-chlorophyll protein complex (PerCP). Optimal compensation and gain settings were determined for each sample based on unstained and single stained samples. Viable cells were gated according to forward scatter and side scatter parameter criteria. At least 20 000 viable cells were acquired from each sample. The number of cells that had proliferated was determined by gating on the lineage-positive, CFSEdim subset. Data were collected with a FACSCalibur flow cytometer and analyzed with CellQuest software. A positive response was defined as both a proliferating fraction (percentage of CFSEdim cells) greater than 1% and a stimulation index (ratio of CFSEdim cell percentage in cultures with antigen to CFSEdim cell percentage in control cultures) of two or more. This combination of criteria has already been validated as stringent in other studies [16,17].

Statistical analysis

Statistical significance was determined using the non-parametric Wilcoxon matched pairs test or the Friedman test to compare data sets of paired groups. All calculations were performed using GraphPad Prism 5 for Windows (GraphPad Software, San Diego, CA).

Results

Elution of Band 3 binding site in CLL cells by acidic pH

In a previous study we showed that treatment of CLL cells with trypsin or proteinase K inhibits Band 3 binding, which suggests the involvement of a membrane protein in Band 3 recognition [4]. As an initial approach to discriminate between integral and peripheral membrane proteins in Band 3 recognition, we used acidic pH to remove extrinsic molecules from CLL cells before the binding assay. Table I summarizes the clinical and phenotypic features of patients with CLL in the present study ($n = 14$). None of these patients showed evidence of AHA nor were treated for at least a period of 6 months from the sample collection date. Leukemic B cells of seven patients from the cohort were exposed to pH 2.5 for 30 s, returned to neutral pH and immediately washed twice with PBS before adding B3N, the Band 3 domain recognized by CLL cells. We found that a brief exposure of leukemic cells to acidic pH significantly reduced their capacity to recognize B3N (Figure 1). In contrast, binding of specific antibodies directed against integral membrane proteins of CLL cells (namely CD19, CD5 or CD25, Supplementary Figure 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.957205), was not affected. These results suggest that the binding of B3N to CLL cells mainly depends on extrinsic molecules that can be eluted with low pH.

Acid elution impairs T cell proliferation induced by Band 3-pulsed CLL cells

We previously reported that, if appropriately stimulated, CLL cells can take up and present Band 3 to autologous CD4 + T cells, inducing their proliferation [4]. This ability could allow the neoplastic clone to trigger the autoaggressive process against erythrocytes. We investigated whether exposure to low pH could reduce the capacity of CLL cells to stimulate CD4 + T cells. To this aim, we purified two cell fractions from PBMCs: CLL cells as antigen presenting cells and T cells as responding cells. CLL cells ($>99\%$ pure as assessed by FACS) were cultured overnight with CD40L-transfected fibroblasts to induce their activation, and then a proportion of CLL cells were exposed to pH 2.5 before being pulsed with Band 3. Finally, Band 3-pulsed activated CLL

Table I. Clinical and phenotypical characteristics of patients with CLL enrolled in this study.

Patient ID	Gender	Age	WBC ($\times 10^9$/L)	Binet stage	CD5 + CD19+ (%)	CD38+ (%)	ZAP-70+ (%)
1	M	61	25	A	96	1	23
2	F	72	29.7	B	93	55	18
3	F	76	19	A	84	9	19
4	M	71	16.5	B	87	54	3
5	M	45	125	C	95	38	84
6	M	68	23.8	B	58	5	10
7	M	50	90	C	94	1	9
8	M	73	60.9	A	89	92	1
9	M	67	17	A	94	1	1
10	F	75	50	B	89	0.3	69
11	F	63	179	B	94	90	25
12	M	56	69.4	A	94	1	ND
13	M	69	200	A	92	6	ND
14	M	64	110	B	91	4	93

CLL, chronic lymphocytic leukemia; WBC, white blood cells; ND, not done.

cells were incubated with CFSE-labeled T cells for 5 days. Supporting our previous results, we found that CD40L-activated CLL cells of seven patients from the cohort pulsed with Band 3 induced proliferation of CD4 + T cells in around 40% of the samples evaluated [Figure 2(A), right panel]. Interestingly, acid elution of CLL cells before being pulsed with Band 3 completely abrogated their capacity to induce T cell proliferation. This impairment in leukemic cells' ability to present Band 3 could not be ascribed to a general dysfunction induced by pH 2.5 exposure, since the treatment neither altered the expression of co-stimulatory molecules CD80 and CD86 [Figure 2(B)] nor impaired CLL cell capacity to stimulate allogeneic T cells in a mixed lymphocyte reaction [Figure 2(C)].

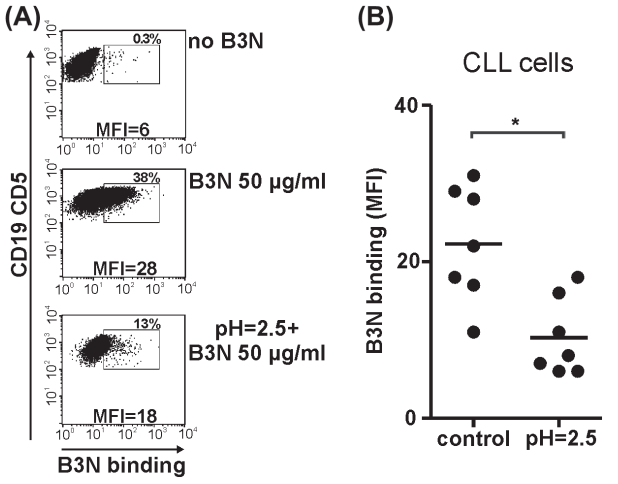

Figure 1. B3N binding to CLL cells depends on non-integral membrane proteins. PBMCs (5×10^5) from patients with CLL were incubated for 30 min at 4°C with B3N (50 µg/mL) and washed three times before the addition of mouse polyclonal anti-B3N antibody. FITC conjugated anti-mouse IgG was used as secondary antibody and binding was analyzed by FACS. Viable leukemic cells were discriminated by forward-scattering gating and CD19 CD5 labeling. When indicated, cells were exposed to pH = 2.5 for 30 s, neutralized and rapidly washed three times before performing the B3N binding assay. (A) Results from a representative patient are shown. Data are expressed as the mean fluorescence intensity (MFI) of B3N binding and as percentages of positive cells settled according to the control condition, in which no B3N was added. (B) Results from a cohort of seven patients are shown as MFI. *$p < 0.05$, control vs. pH = 2.5 condition.

Identification of HMGN2 as binding protein to Band 3

We reasoned that if acidic treatment decreases the binding of B3N to leukemic B cells, the molecule(s) implicated in this interaction would be released from the cell membrane as a consequence of low pH exposure and, hence, supernatants of the acidic treatment experiments would become a useful tool to identify putative ligands of Band 3. With this in mind, we obtained supernatants from 100×10^6 CLL cells exposed to pH 2.5 and, after neutralization, loaded them onto an agarose-B3N affinity column. Figure 3(A) depicts the eluted peak at 280 nm. The evaluation of this fraction by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed a protein with an apparent molecular weight of 17 kDa, which was in turn identified by MALDI-TOF/TOF as HMGN2 of *Homo sapiens* [Figure 3(B)]. Comparable results were obtained with three different CLL samples. Interaction between B3N and HMGN2 was corroborated by ELISA as shown in Figure 3(C). These results identified HMGN2 as a putative partner of B3N implicated in Band 3 binding to CLL cells.

CLL cells present HMGN2 at membrane

Since HMGN2 was not previously reported to be located at the cell membrane, we used a specific antibody to evaluate its presence on CLL cells by FACS and fluorescence microscopy. Figure 4 shows that CLL cells of seven patients from the cohort (CD19 + CD5+) but not T lymphocytes (CD3+) presented HMGN2 at their surface. This is relevant, since we have previously reported that neither T lymphocytes nor natural killer (NK) cells can bind Band 3 [4]. In line with these results, HMGN2 was also found in Daudi cells, a human lymphoma-derived B cell line with the capacity of binding Band 3 [Supplementary Figure 2(A) to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.957205]. In Daudi cells we were also able to corroborate that acid elution reduced the binding of B3N [Supplementary Figure 2(B) to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.957205], without affecting the expression of CD19, cell viability or proliferative capacity [Supplementary Figure 2(C) to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.957205].

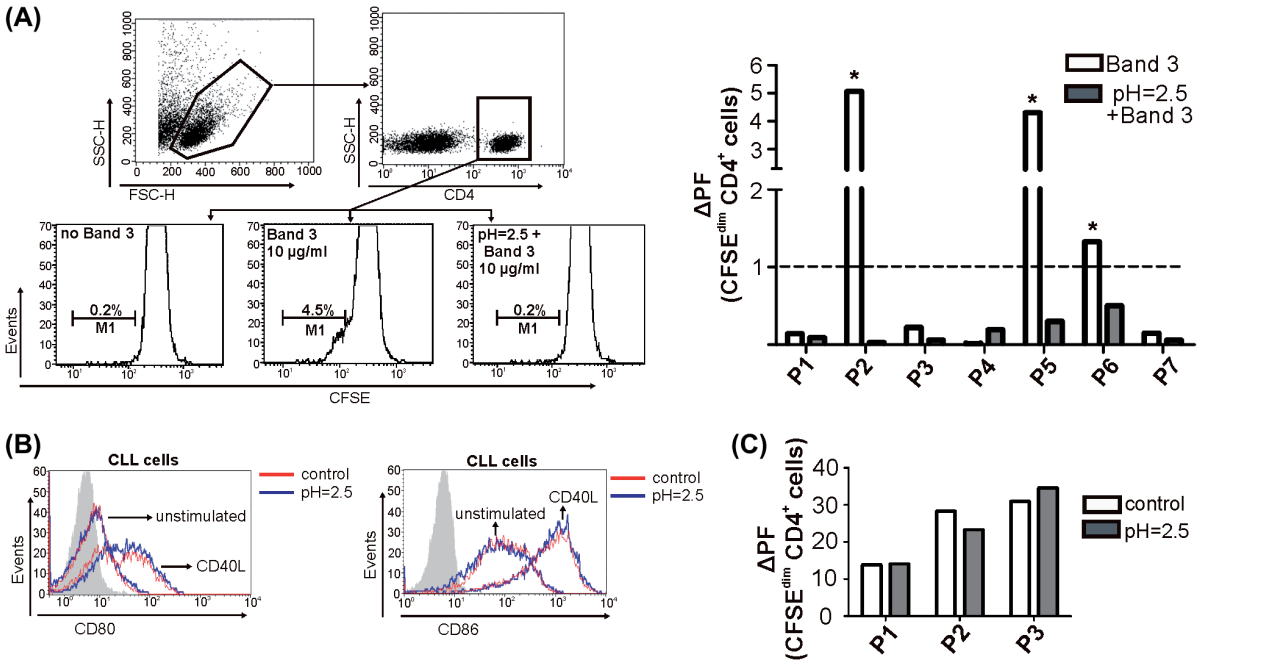

Figure 2. Removal of non-integral membrane proteins impairs T cell proliferation induced by Band 3-pulsed activated CLL cells. Highly purified CLL cells (> 99%) were cultured for 24 h over gamma-irradiated CD40L-transfected fibroblasts, removed from the adherent layer and incubated in presence or absence of Band 3 for 6 h. When indicated, CD40L-activated CLL cells were exposed to pH = 2.5 for 30 s, rapidly neutralized and washed three times in RPMI before incubation with Band 3. After culture, cells were washed twice in RPMI to remove the erythrocyte antigen. Autologous purified T cells were labeled with CFSE and co-cultured with Band 3-pulsed CLL cells at 1:1 ratio at 10^6 cells/mL for 5 days. Finally, cells were stained for CD4 and T cell proliferation was evaluated by FACS using the CFSE dilution method. (A) T cell proliferative response to Band 3-pulsed CLL cells. Shown are representative histograms from a responding patient (left panel). Percentage of CFSEdim cells was calculated on CD4 + viable cells. Right panel depicts proliferative responses of CD4 + T cells induced by CLL cells pulsed with Band 3 at neutral pH (white bars) or pH = 2.5 (black bars). Data from seven samples evaluated are plotted as Δ proliferation fraction (ΔPF). A response with ΔPF of at least 1% and stimulation index of at least 2.0 was considered positive (indicated with *). (B) Acid elution does not modify the expression of co-stimulatory molecules CD80 and CD86 in unstimulated and CD40L-stimulated CLL cells. Purified CLL cells treated as described above were evaluated for expression of CD80 and CD86 by FACS. Representative histograms are shown (isotype controls are depicted in gray). (C) Acid elution does not impair CLL cell capacity to stimulate allogeneic T cell proliferation. Purified CLL cells exposed to pH = 2.5 (black bars) or neutral pH (white bars) as previously described were co-cultured with CFSE-labeled allogeneic T cells from healthy donors at a 1:10 ratio at 10^6 cells/mL for 5 days. Data from three CLL samples are plotted as ΔPF.

Feng *et al.* [18] reported that stimulation of human leukocytes with interleukin-2 (IL-2) induced the release of HMGN2 to the extracellular environment. Taking these previous results into account, we evaluated whether incubation of CLL cells with IL-2 or four other relevant cytokines in CLL biology could modify the levels of HMGN2 at the cell membrane. We found that only interferon α (IFNα) was able to increase both the binding of B3N and HMGN2 surface expression (Supplementary Figure 3 to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.957205), while IL-2, IL-10, IL-6 or transforming growth factor β (TGFβ) had no effect (data not shown).

Since acid elution allowed us to identify HMGN2 as a binding site of Band 3 in the CLL cell membrane, we reasoned that the same treatment should decrease its membrane localization. Therefore, we exposed CLL cells to pH 2.5 for 30 s, returned to neutral pH and evaluated surface HMGN2 by FACS (Figure 5). As expected, we found that a brief exposure of cells to low pH almost completely abrogated the presence of HMGN2 at the cell membrane.

Discussion

In the present study we identified HMGN2 as a major binding site for the erythrocyte antigen Band 3 on the CLL cell membrane. We also found that the removal of HMGN2 from the CLL cell surface abrogates not only the capacity to bind Band 3 but also to induce specific CD4 + T cell proliferation. Therefore, the presence of HMGN2 as an extrinsic molecule on the CLL cell membrane might be involved in the initiation of AHA associated with CLL.

HMGN2 belongs to a family of nuclear proteins that bind to nucleosomes and modulate the structure and function of chromatin [19,20]. While HMGNs are mainly intranuclear proteins, they can also have extracellular activities. Thus, HMGN1 was shown to act as an endogenous mediator capable of inducing dendritic cell maturation via Toll-like receptor 4 interaction [21]. By using knock-out mice and bone marrow chimeras, it was found that HMGN1 was secreted by cells other than leukocytes at the site of immunization and was critical for the induction of antigen-specific responses. On the other hand, Feng *et al.* [18] reported that HMGN2 can be released from IL-2-activated lymphocytes and exerts a potent antimicrobial activity against bacteria, fungi and virus [22–24]. None of these reports, however, evaluated the stable expression of HMGN proteins at the cell membrane. Our results show that malignant B cells from patients with CLL and from the lymphoblastoid Daudi cell line present significant levels of surface HMGN2 while T lymphocytes do not. Two questions arise from these findings: what is the source of

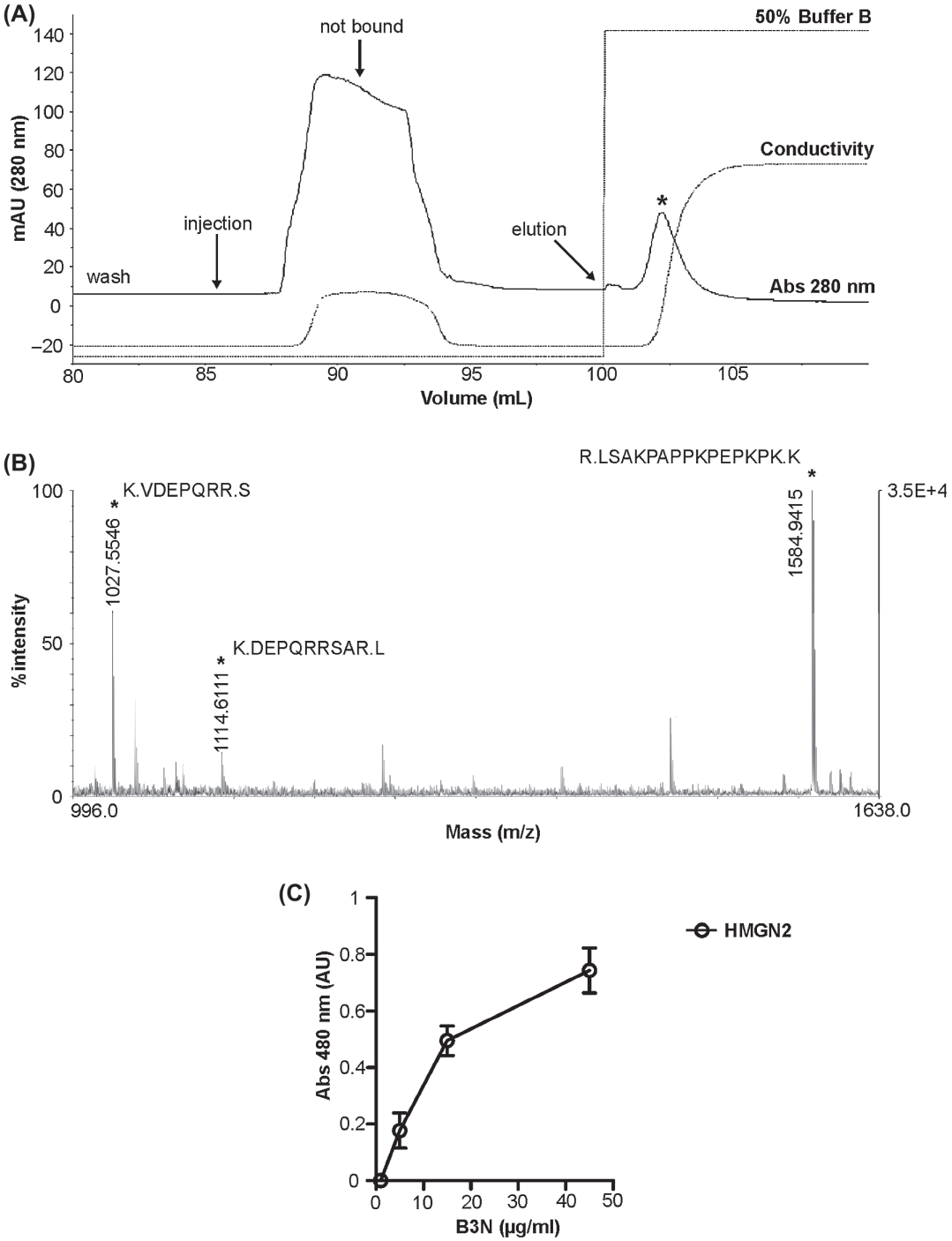

Figure 3. Identification of HMGN2 as a B3N ligand. (A) PBMCs (100×10^6) from three patients with CLL were incubated for 30 s in pH = 2.5 buffer, neutralized and rapidly centrifuged. Supernatants were collected and injected into an agarose-B3N affinity column. Elution of retained proteins was performed by ionic strength and posterior acidic pH. Shown is a representative chromatogram of the experiences, depicting a single peak (*) eluted with 0.5 M NaCl. Buffer B = 20 mM Tris pH 7.5, 1 M NaCl. (B) Mass spectra obtained when the sample corresponding to the peak (*) shown in (A) was submitted to MALDI-TOF/TOF analyses. Sequence query search of the trypsinized peptides included oxidation (M) as variable modification. Mass values: monoisotopic. Protein mass: unrestricted. Peptide mass tolerance: ± 0.05 Da, fragment mass tolerance: ± 0.3 Da. HMGN2 protein score: 82; pepetide score: 56. Protein sequence coverage: 28%. (C) HMGN2 or BSA (0.5 µg/well) was coated for 18 h using ELISA 96-well plates, washed five times and incubated for 2 h in PBS 3% BSA at room temperature. Plate was washed again and B3N was added at the indicated concentrations in triplicate. After the last washes, polyclonal mouse anti-B3N serum was added. Interaction was revealed using an HRP-conjugated anti-mouse IgG and TMB substrate. Detection was assessed using a 450 nm filter. Shown is a representative experiment ($n = 3$).

HMGN2 and how does it bind to the cell membrane? Regarding the source of surface HMGN2, leukemic B cells could actively produce and export the protein or, alternatively, they could bind HMGN2 released by necrotic or damaged cells. Both types of mechanisms have been well documented for HMGB1, an intranuclear DNA binding protein that acts as an alarmin and is responsible for the induction of pro-inflammatory cytokines [25,26]. Our results showing that culture of CLL cells with IFNα increased the expression of HMGN2 but markedly inhibited cell death (Supplementary Figure 2

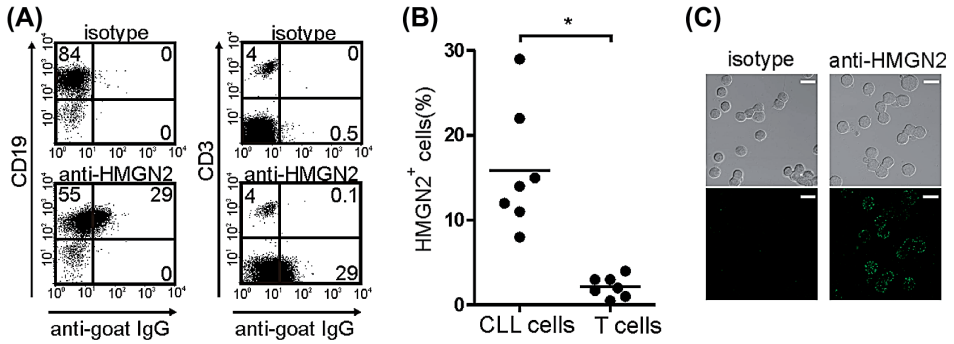

Figure 4. CLL cells present HMGN2 at the cell surface. Freshly isolated PBMCs (5×10^5) from patients with CLL were incubated with goat anti-human HMGN2 IgG or irrelevant goat IgG (10 μg/mL) for 30 min at 4°C. Cells were washed three times and anti-goat IgG labeled with Alexa-488 was added for an additional 30 min. Leukemic cells were discriminated as CD19 + and T lymphocytes as CD3+. (A) Representative dot plots from seven CLL samples evaluated by FACS. Numbers represent the percentage of cells within each quadrant. (B) Percentages of CLL and T cells positive for surface HMGN2. $^*p < 0.05$, Wilcoxon matched pairs test. (C) Representative fluorescent micrographs of PBMCs (>97% leukemic cells) stained for HMGN2 surface expression. White bars = 10 μm.

to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.957205) support the former possibility. Even though the source of surface HMGN2 remains an open issue, the enhancing effect of IFNα on surface HMGN2 and Band 3 binding is an interesting observation in the context of AHA. In fact, AHA is a well-known side effect in patients treated with IFNα for hematological diseases, including CLL [27].

With regard to how HMGN2 binds to the cell surface, we attempted to gain structural insights using computational methods based on fold recognition and remote homology searches against structurally characterized proteins. However, we were not able to identify a folding pattern or remote homologs with known structure. Indeed, using prediction methods for disordered proteins [28] indicates that HMGN2 is an intrinsically disordered protein, precluding the possibility of obtaining structural information. In light of this we can only speculate that HMNG2 interacts with a yet unidentified membrane protein or with a non-proteinaceous membrane component. As for the second possibility, it should be mentioned that the proline rich antibiotic peptide Bac7, which shares some similarity with the central region of HMNG2, has been reported to be retained on the membrane surface, interacting with phospholipid heads [29]. We modeled this last possibility employing a previously reported technique [30] by using a random conformation of HMNG2. As shown in Figure 6, this last possibility would allow for the simultaneous interaction of HMNG2 with the membrane and other partners, as in the case of B3N through the HMGN2

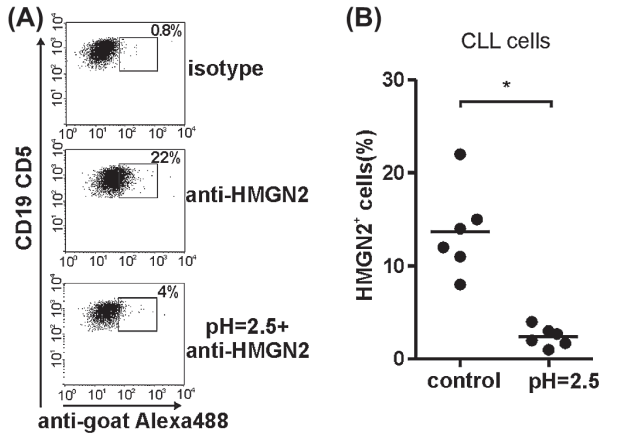

Figure 5. Acid elution removes HMGN2 from the cell membrane. Freshly isolated PBMCs (5×10^5) from patients with CLL were incubated with goat anti-human HMGN2 IgG or irrelevant goat IgG (10 μg/mL) for 30 min at 4°C. When indicated, cells were exposed to pH = 2.5 for 30 s, rapidly neutralized and washed three times in PBS before the addition of anti-HMGN2 Ig or isotype control. Cells were washed three times and anti-goat IgG labeled with Alexa-488 was added for an additional 30 min. Data were analyzed by FACS in viable leukemic cells discriminated by forward-scattering and CD19 + CD5+ gating. (A) Results from a representative patient with CLL are shown. Numbers represent the percentage of HMGN2 + CLL cells. (B) Percentages of CLL cells positive for surface HMGN2 in control conditions and after acid elution. $^*p < 0.05$, control vs. pH = 2.5, $n = 6$, Wilcoxon matched pairs test.

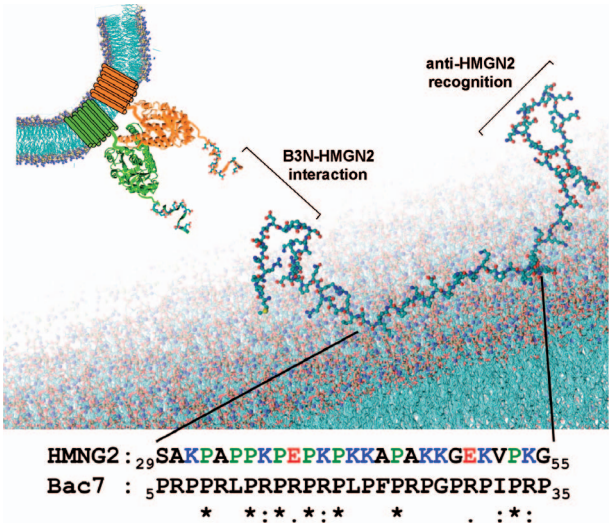

Figure 6. Schematic modeling of a putative interaction between B3N and surface HMGN2. Shown is a conformer of molecular dynamics simulation of HNMG2 bound to a membrane patch. The region in contact with the membrane roughly corresponds to the region detailed in the alignment. Bottom: Sequence alignment of the proline rich region of HMNG2 and Bac7. Proline, lysine and glutamic acids in HMNG2 are colored in green, blue and red, respectively. Top: negatively charged N-terminal domain of Band 3 exposed in inverted red blood cell vesicles could interact with positively charged N-terminal domain of HMGN2. Antibody against C-terminal region of HMGN2 would also be capable of recognizing its antigen in the proposed scheme.

N-terminal domain, or the antibody used in this study, which recognizes the HMGN2 C-terminal domain. Still, further biochemical and structural characterization of HMGN2 interaction with the cell membrane and B3N are required to support this proposal.

In conclusion, our results demonstrate that HMGN2 can be found as an external peripheral membrane protein in leukemic B cells and is a major ligand for erythrocyte Band 3. We propose that expression of HMGN2 enables CLL cells to act as aberrant presenting cells for erythrocyte autoantigens and thus might be related to the initiation of AHA secondary to CLL.

Acknowledgements

We thank Beatriz Loria for technical assistance. We also wish to express our gratitude to the patients who consented to participate in this study.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

This work was partially funded by FOCEM (MERCOSUR Structural Convergence Fund), COF 03/11.

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Supplementary material available online

Supplementary Figures showing further data