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Chronic Lymphocytic Leukemia Cells Bind and Present the Erythrocyte Protein Band 3: Possible Role as Initiators of Autoimmune Hemolytic Anemia[1]

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The mechanisms underlying the frequent association between chronic lymphocytic leukemia (CLL) and autoimmune hemolytic anemia are currently unclear. The erythrocyte protein band 3 (B3) is one of the most frequently targeted Ags in autoimmune hemolytic anemia. In this study, we show that CLL cells specifically recognize B3 through a still unidentified receptor. B3 interaction with CLL cells involves the recognition of its N-terminal domain and leads to its internalization. Interestingly, when binding of erythrocyte-derived vesicles as found physiologically in blood was assessed, we observed that CLL cells could only interact with inside-out vesicles, being this interaction strongly dependent on the recognition of the N-terminal portion of B3. We then examined T cell responses to B3 using circulating CLL cells as APCs. Resting B3-pulsed CLL cells were unable to induce T cell proliferation. However, when deficient costimulation was overcome by CD40 engagement, B3-pulsed CLL cells were capable of activating $CD4^+$ T cells in a HLA-DR-dependent fashion. Therefore, our work shows that CLL cells can specifically bind, capture, and present B3 to T cells when in an activated state, an ability that could allow the neoplastic clone to trigger the autoaggressive process against erythrocytes. *The Journal of Immunology,* **2008, 181: 3674–3683.**

Chronic lymphocytic leukemia (CLL)[3] is the most prevalent form of adult leukemia in the Western hemisphere and is characterized by the slow buildup of a B cell clone with mature phenotype (1, 2). It is now recognized that the disease may follow two different pathways: either take an indolent course, with a normal life expectancy (patients die with CLL, rather than from the disease), or show an aggressive behavior, with a considerably shortened survival (3–5 years). The clinical heterogeneity of CLL correlates with certain features of the leukemic cell that have emerged as prognostic factors of the disease. Unmutated Ig variable region and the expression of CD38 and/or ZAP-70 are considered the most useful tools in identifying aggressive CLL. Patients with progressive disease usually die from infectious complications due to the immunosuppression induced by this neoplasia or the autoimmune phenomena that characterize the most advanced disease stages (3).

Among the autoimmune derangements that arise in the context of CLL, autoimmune hemolytic anemia (AHA) is the most frequent, and usually of the warm, IgG-mediated type (4, 5). In this process, autoantibodies targeting surface membrane proteins lead erythrocytes to an accelerated destruction by spleen macrophages and Kupffer cells. Because AHA in general most commonly occurs in the setting of CLL, a cause/effect relationship has long been sought (6). The initial assumption was that the neoplastic cells themselves were responsible for autoantibody production. This hypothesis was later discarded after Ab analysis from erythrocyte eluates showed a polyclonal nature and found no differences from those in AHA secondary to systemic lupus erythematosus (7), an autoimmune systemic disease in which no abnormal B cell clone has been described. Therefore, anti-erythrocyte Abs, which are of the IgG isotype and exhibit affinity maturation, must be produced by normal, autoreactive B cells in a T cell-dependent manner.

As in most autoimmune phenomena, T cell activation is the crucial event of AHA pathogenesis. But what circumstances could favor autoreactive T cell activation in CLL patients and not in healthy individuals? It is very striking that this neoplasia frequently associates to autoimmunity against blood cell Ags (mostly erythrocyte proteins, and secondarily, platelets in the form of immune thrombocytopenic purpura), but not to other autoimmune phenomena and that splenomegalia due to leukemic cell infiltration often accompanies AHA in the advanced clinical forms of the disease (4, 8). RBC accumulate in the spleen where they are destroyed by phagocytes and by the repeated passages through red pulp sinusoids, making the splenic stroma a rich source of the targeted Ags. Hall et al. (9) previously reported that one of the most prevalent Ags in AHA, the Rh group, could be presented by CLL cells to autoreactive T lymphocytes inducing proliferative responses. Thus, CLL cells acting as aberrant APCs would be responsible for initiating the autoimmune response. However, it is

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[3] Abbreviations used in this paper: CLL, chronic lymphocytic leukemia; AHA, autoimmune hemolytic anemia; B3, band 3; RSOV, right-side out vesicle; IOV, inside-out vesicle; sIg, surface Ig; MFI, mean fluorescence intensity.

unclear how CLL cells could take up and process this protein considering that they are not efficient at fluid-phase endocytosis nor they express Rh-specific BCR (10, 11). Besides the Rh group, the anion exchanger also known as band 3 (B3) (12), is a frequent targeted Ag both in human and murine AHA (7, 13–16). When modified on *Plasmodium*-infected erythrocytes, B3 can be recognized by the scavenger receptor CD36 present on the monocyte membrane (17, 18). Taking into consideration that CD36 is also expressed by CLL cells (19), in the present study we evaluated whether the leukemic cells were able to bind soluble B3 as an initial step to take up and present this autoantigen to T lymphocytes. Our findings show that CLL cells specifically recognize the N-terminal portion of B3 through a binding site different from CD36. In addition, we found that, upon appropriate costimulation, B3-pulsed CLL cells can induce $CD4^+$ T cell proliferation by a HLA-DR-dependent mechanism, indicating that Ag presentation is involved.

Materials and Methods

Patients

Sterile heparinized peripheral blood was obtained from 35 patients with B-CLL (age range: 51 to 78), who were either untreated or had not received cytoreductive chemotherapy for at least 3 mo before investigation. B-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 used in this study were obtained after informed consent in accordance with the Declaration of Helsinki and with Institutional Review Board approval from the National Academy of Medicine, Buenos Aires.

Reagents and Abs

All chemicals, enzymes, and detergents used were from Sigma-Aldrich unless otherwise specified. The Abs anti-CD3, -CD4, -CD5, -CD8, -CD14, -CD16, -CD19, -CD38, -CD56, -CD80, -CD86, IgM, κ, and λ Ig light chains and ZAP-70 (clone 1E7.2) were from BD Pharmingen. Anti-B3N Ab was from GeneTex (clone BIII-136). A mAb (clone 10D2) against an extracellular epitope of the membrane domain of B3 was raised by immunizing mice with human erythrocytes and characterized by ELISA and Western blotting.

Proteins

B3 was purified from pooled erythrocytes of healthy volunteers by a modification of the method of Casey (20). In brief, erythrocyte ghosts were stripped off peripheral proteins and solubilized in deoxycholate 1% m/v in low ionic strength buffer to selectively act on B3 and not the glycophorins (21). Excess detergent and contaminating proteins were removed by DEAE anion exchange chromatography and dialysis. Finally, the protein was concentrated and stored at −80°C. Spectrin was obtained as a byproduct of B3 purification and treated likewise. Both proteins were conjugated to FITC or NHS-LC-, NHS-SS-biotin (Pierce) according to the manufacturer's instructions.

Vector pET21a containing the cytoplasmic N-terminal domain of B3 (B3N) with C terminus His6 tag (provided by Dr. Yu Ding) was cloned into BL21 (D3) pLys for its expression, as previously described (22). 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 Resourse Q column (GE Healthcare). Purified protein was concentrated and stored at −80 °C.

Preparation of erythrocyte membrane vesicles

Right-side out (RSOV) and inside-out vesicles (IOV) were prepared from pooled erythrocytes from healthy donors as described by Hargreaves (23). In brief, for RSOV preparation erythrocyte ghosts were forced several times through a 27-gauge hypodermic needle in 5 mM phosphate buffer (pH 8) containing protease inhibitors. IOV were obtained by incubating erythrocyte ghosts with 0.1 mM EDTA (pH 8) for 30 min at 37°C and finally forcing the membranes through a 27-gauge needle. Both preparations were washed with PBS before use. For biotin labeling, the instructions from Pierce for cell tagging were followed. Membrane orientation was assessed by FACS with anti-glycophorin A Ab (exofacial epitope, BD Pharmingen) and anti-B3 Ab (endofacial epitope, clone BIII-136 from GeneTex).

Isolation of PBMC and preparation of cell fractions

PBMC were isolated from fresh blood samples by centrifugation over a Ficoll-Hypaque layer (Lymphoprep, Nycomed Pharma). T cell-enriched fractions (T cells) were prepared by negative selection with anti CD14, CD16, CD56, and CD19 and magnetic beads (Pierce), according to the manufacturer's instructions. CLL cell-enriched fractions (CLL cells) used as APCs were prepared by negative selection with anti-CD2, -CD3, -CD14, -CD56, and -CD16 by the same procedure. T cell purity was >95% and CLL cell purity was always 99% or greater, assayed by FACS.

Evaluation of ZAP-70 and CD38 expression in CLL cells by FACS

ZAP-70 expression was evaluated by FACS as previously described (24). In brief, 0.5×10^6 PBMC were stained for 20 min at 4°C with anti-CD19PerCP, anti-CD3PE, and anti-CD56PE. After washing twice with PBS-0.5% BSA, cells were fixed and permeabilized with Fix and Perm kit (Caltag Laboratories) according to the manufacturer's intructions. Then, 2 μg of FITC-conjugated anti-ZAP-70 Ab was added and cells were incubated for an additional 20 min at 4°C. Analysis of samples was conducted using CellQuest software (BD Biosciences). Lymphocytes were gated to exclude debris, monocytes and doublets. $CD3^+CD56^+$ cells (T and NK cells) were used as an internal control for ZAP-70 expression. The percentage of CLL cells expressing CD38 was determined as previously described (2).

Binding and endocytosis

The binding of biotinylated or FITC-conjugated B3 to different cells types was assayed by FACS. Cells were incubated on ice with different protein concentrations for 20 min in PBS BSA 0.5% and then washed three times with the same buffer before tagging with streptavidin-FITC when using biotin. To discriminate the different leukocyte populations, cells were further incubated with conjugated Abs (anti-CD3PerCP, anti-CD19PE, anti-CD14PE, anti-CD56PE) and immediately acquired. Endocytosis was measured by performing B3-SS-biotin binding as described above on purified CLL cells (>98% purity) and then incubating the cells at 37°C for 45 min to allow protein internalization. Biotin remaining on the cell surface was cleaved off by three rounds of MesNa 150 mM treatment, 30 min each, and the residual MesNa was quenched for 15 min with 5 mg/ml iodoacetamide in PBS. After washing one more time in PBS, cells were lysed in PBS Triton X100 1% with protease inhibitors. Samples were subjected to immunoblot analysis with streptavidin-HRP (DakoCytomation) and quantified with Gel-Pro Analyzer software (Media Cybernetics). For erythrocyte vesicle binding, cells were incubated with membrane suspensions and treated in the same way as for soluble B3 binding.

Cells and culture

PBMC or PBMC-derived cells were cultured at the concentration of $1–1.25 \times 10^6$ cells/ml in RPMI 1640 (Life Technologies) supplemented with 10% pooled heat-inactivated human AB serum, 100 μg/ml streptomycin, 100 U/ml penicillin, and $5 \times 10^-$ M 2-ME, in a humidified atmosphere of 5% CO_2/95% air. Murine fibroblasts stably transfected with a human CD40L cDNA were provided by Dr. Claire Hivroz from Institut Curie, Paris.

T cell proliferation assays

To evaluate proliferation of PBMC, cells were cultured in quadruplicate in 96-well U-bottom plates with B3 at 10 μg/ml for 5–7 days, and [^{3}H]thymidine incorporation was assayed for the last 18 h of culture. To evaluate proliferation of purified T cells, 10^6 T cells were seeded in 1 ml culture medium in 24- or 48-well plates immediately after enrichment, while CLL cells were seeded onto gamma-irradiated CD40L fibroblast monolayers for overnight stimulation, then nonadherent cells were picked up and pulsed for 6 h with 10 μg/ml B3 in new wells for Ag uptake and to allow contaminating fibroblasts to re-adhere at the same time. Nonadherent cells were gently picked up, washed three times with culture medium to remove unbound Ag and gamma irradiated (30 Gy). Finally, 10^6 B3-pulsed CLL cells were added to T cells and cultured for 5–7 days. Aliquots were taken at defined timepoints and pulsed with [^{3}H]thymidine in quadruplicate for 18 h. For analysis, data are presented as stimulation indexes, calculated as the ratio of cpm of cultures in the presence of Ag to cpm of control cultures. A positive response was defined as an increase greater than the mean cpm plus three SD of control samples, which was considered significant and has been previously validated (25).

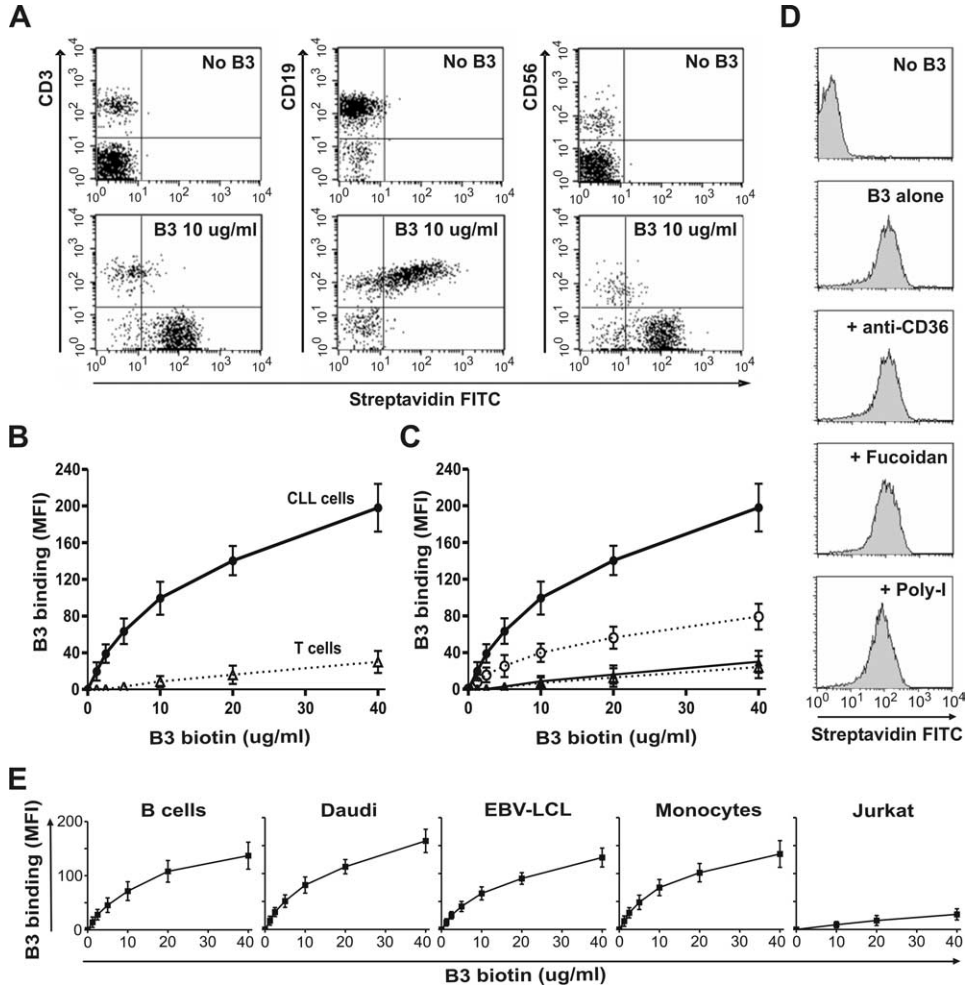

FIGURE 1. B3 specifically binds to CLL cells. PBMC from leukemic patients were incubated with biotin-conjugated B3 in the concentration range of 0–40 μg/ml and washed before tagging with streptavidin-FITC. Cell populations were discriminated by forward-scattering gating and CD3, CD19, or CD56 labeling and analyzed by FACS. *A*, Dot plots of $CD3^+$, $CD19^+$, and $CD56^+$ population bindings with B3 10 μg/ml from a representative sample ($n = 35$). Of note, CLL cells ($CD19^+CD5^+$) comprised >90% of the marker-negative population for CD3 and CD56 dot plots and >95% of the $CD19^+$ population. *B*, Total B3 binding curve for CLL (●) and T cells (Δ). Data are expressed as the mean fluorescence intensity (MFI) of biotin-conjugated B3 revealed with streptavidin-FITC. Values are the mean ± SEM from five patients analyzed. *C*, Effect of excess unlabelled B3 (400 μg/ml) on the total B3 binding curve. Values are the mean ± SEM from three patients analyzed. CLL cells with (○) or without (●) cold B3 and T cells, with (□) or without (■) cold B3. *D*, Effect of a blocking anti-CD36 Ab and two competing scavenger receptor ligands on B3 binding to CLL cells. PBMC were incubated with binding medium alone, Ab at 10 μg/ml, 100 μg/ml fucoidan, or 100 μg/ml poly-inosine (poly-I) for 30 min in the cold and then biotin-conjugated B3 was added at 10 μg/ml final concentration without washing. Experiment then proceeded as previous binding experiments. Representative histograms ($n = 3$) corresponding to the $CD19^+$ cell population are shown. *E*, Cells were incubated with biotin-conjugated B3 as previously described for CLL samples and total binding curves were obtained for $CD19^+$ (B cells) and $CD14^+$ (monocytes) cells in PBMC from six healthy donors; Daudi cells and EBV-immortalized B cell lines from two healthy donors (EBV-LCL); and Jurkat cells. Daudi and Jurkat cell lines were assayed in triplicate and MFI values for all cases are the mean ± SEM of all samples or replicates analyzed.

Assessment of cell proliferation by flow cytometry

To evaluate proliferation of T cell subpopulations, the CFSE dilution assay was used (26). T cells (10^7/ml) in PBS were incubated at 37°C for 10 min with 0.5 μM CFSE (Invitrogen). Staining was terminated by adding RPMI 1640 with 5% serum. Cells were thoroughly washed in PBS. Stained T cells and activated CLL cells (either pulsed with B3 or medium alone) were cocultured at 1:1 ratio and 10^6 cells/ml for 5 days. Cells were harvested, washed in PBS plus 0.5% BSA, and stained on ice with anti-human CD4-PerCP and anti-human CD8-PE mAbs. 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 parameters criteria. At least 10,000 viable cells were acquired from each sample. The number of cells that had proliferated was determined by gating on the lineage-positive, $CFSE^{dim}$ 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 $CFSE^{dim}$ cells) greater than 1% and a stimulation index (ratio of $CFSE^{dim}$ cell percentage in cultures with Ag to $CFSE^{dim}$ cell percentage in control cultures) of two or more. This combination of criteria has already been validated as stringent in other studies (26, 27).

Blockade of HLA class II-mediated responses

HLA-DR-blocking Ab (clone L243) was provided by Dr. P. Benaroch, from Institut Curie, Paris. The mAb was purified from hybridoma supernatant by protein-G-Sepharose chromatography and dialyzed against PBS. It was added to cell cultures at a final concentration of 2.5 μg/ml.

Statistical analysis

Differences in B3 binding under different conditions were compared with native B3 binding by using Student's *t* test. Differences in CD80 and CD86 expression and B3 binding between resting and CD40L-activated CLL cells were also evaluated by Student's *t* test. All calculations were performed with GraphPad Prism 4 for Windows (GraphPad Software).

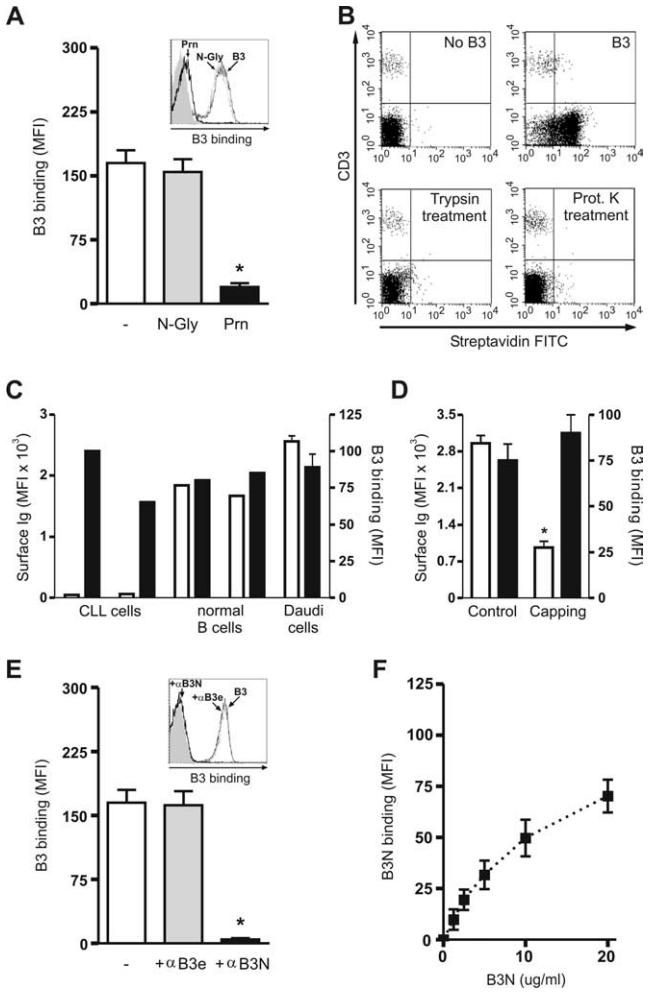

FIGURE 2. Characterization of B3 binding site on B cells. *A*, Effect of enzymatic deglycosylation or proteolisis of B3 on protein binding. Digestions with PNGase F (N-Gly and dotted-line histogram) or pronase (Prn and thick-line histogram) were performed according to the manufacturer's instructions. PBMC from CLL patients were incubated with naive or digested B3 (10 μg/ml) and cell populations were discriminated with specific Abs. Results shown correspond to $CD19^+$ cells. Bars represent mean MFI $\pm$ SEM, $n = 3$. *Inset* shows a representative histogram. *B*, Effect of mild enzymatic pretreatment of CLL cells on B3 binding (representative example, $n = 4$). Cells were either left untreated (*top right*) or incubated with proteinase K (*bottom left*) or trypsin (*bottom right*). Enzymes were inhibited with phenylmethanesulphonylfluoride after a 5-min incubation period and cells were thoroughly washed with binding buffer before addition of B3. *Top left,* Control cells with no B3. Of note, enzymatic digestion was mild enough not to modify CD3 labeling. *C*, Comparison between sIg expression (□, detected by κ-λ L chain staining) and B3 binding at 10 μg/ml (■) in different B cell populations: leukemic cells from two CLL samples, B cells from two healthy donors, and Daudi cells. *D*, Effect of anti-IgM-induced sIg capping on B3 binding in Daudi cells. Cells were treated with 10 μg/ml $F(ab')_2$ goat anti-human IgM in the cold for 15 min, washed, and incubated at 37°C for 2 h in the presence of 5 μg/ml rabbit anti-goat IgG. B3 binding at 10 μg/ml (■) was performed as previously described with control and capped Daudi cells. sIg expression (□) was detected by PE-conjugated κL chain staining. Results are expressed as the mean MFI $\pm$ SEM, $n = 3$. *E*, Effect of anti-B3 Ab directed against the 200 N-terminal residues (αB3N and thick-line histogram) or against the extracellular portion (αB3e and dotted-line histogram) on B3 binding (empty bar and thin-line histogram). PBMC from CLL patients were incubated with biotin-conjugated B3 (10 μg/ml) in the presence of anti-B3 IgG and cell populations were discriminated with specific Abs. Results shown correspond to $CD19^+$ cells. Abs were used at 20 μg/ml. Bars represent mean MFI $\pm$ SEM, $n = 5$. *Inset* shows a representative histogram. *F*, Total B3N binding curve for CLL cells. Experiment was performed as described for Fig. 1*B* but recombinant, biotin-conjugated B3N was used instead of purified B3. Values are the mean $\pm$ SEM from five patients analyzed. *, Statistically significant difference according to Student's paired *t* test ($p < 0.05$).

Results

CLL cells specifically bind B3

To test the hypothesis that CLL cells could recognize B3 as an initial step to take up and present this autoantigen, we first evaluated B3 binding capacity of peripheral lymphoid cells from CLL samples discriminating between $CD3^+$, $CD56^+$, and $CD19^+CD5^+$ cells by FACS. As shown in Fig. 1*A*, CLL cells bound B3 to a very high extent compared with the negligible levels in the T and NK cell populations. CLL cells detectably bound the protein starting at 0.5 μg/ml with a binding curve showing evidence of saturability (Fig. 1*B*). Binding of B3 appears to be a specific phenomenon since: 1) spectrin and albumin used as control proteins showed no affinity for any cell type evaluated (data not shown) and 2) binding was markedly displaced by excess untagged protein (400 μg/ml) (Fig. 1*C*). Higher cold protein concentrations could not be used due to cell aggregation. Interestingly, neither a blocking anti-CD36 Ab nor competing scavenger ligands for this receptor (fucoidan, poliinosine) (28) had any effect on B3 binding by CLL cells, indicating that CD36 is not involved (Fig. 1*D*). All CLL patients analyzed ($n = 35$) bound B3 with some variability between samples. About half of these samples ($n = 16$) were analyzed for ZAP-70 and CD38 expression and no correlation was observed between B3 binding and prognostic markers (data not shown). Finally, we extended these findings on CLL B-lymphocytes to normal B cells in healthy donors ($n = 8$), EBV-immortalized B cell lines ($n = 2$), and Daudi cells (Fig. 1*E*). Monocytes from both healthy donors and leukemic patients also bound B3. In contrast, Jurkat cells, a T cell line, showed negligible B3 binding, as did their normal counterparts, T lymphocytes from blood samples (Fig. 1, *B* and *E*).

Characterization of B3 binding site on CLL cells

B3 is a glycosylated protein, containing a single complex N-linked oligosaccharide that is attached to Asn in the fourth extracellular loop (12). It has been reported that nucleolin, which is expressed on monocytes and B cells, is capable of recognizing the B3 glycan (29). To elucidate whether this receptor was involved in the binding of B3 by leukemic cells, we proceeded to remove the glycan from soluble B3 by N-glycanase treatment. Effectiveness of deglycosylation was monitored by m.w. shift in SDS-PAGE (30). Deglycosylated B3 bound to CLL cells as efficiently as native B3, showing that the putative receptor does not interact with the glycan (Fig. 2*A*). In contrast, pronase digestion of B3 completely abolished its binding (Fig. 2*A*). Both results suggest that the peptide backbone, not the glycan itself, bears the motif responsible for cell surface binding.

When CLL cells were pretreated with trypsin, B3 binding was strongly inhibited (Fig. 2*B*), suggesting that proteins are involved. Protease digestion was not harsh enough to remove the surface markers CD3, CD19, and CD56 used to discriminate cell populations by FACS, so this reduction cannot be ascribed to lack of sites for nonspecific binding due to total surface protein stripping. Similar results were obtained with proteinase K (Fig. 2*B*). Surface Ig (sIg) appears not to be the putative receptor because there was no correlation between sIg expression level and B3 binding in different B cell populations (Fig. 2*C*). To corroborate this, we used

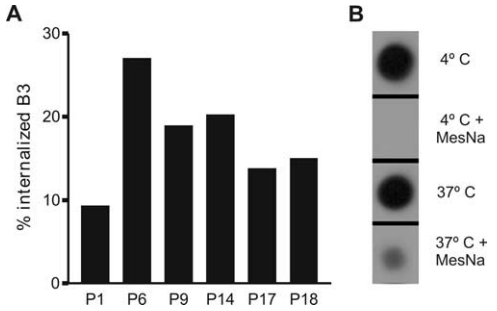

FIGURE 3. Uptake of membrane-bound B3 by CLL cells. Purified CLL cells from five patients were incubated with B3-SS-biotin 10 μg/ml in the cold for 20 min, then extensively washed. Half of the cells were shifted to 37°C for 45 min while the rest remained in the cold. Then, each half was split in two and either treated with MesNa (reducing agent that cleaves SS-biotin) or left untreated. After cell lysis with PBS-Triton X-100 supplemented with protease inhibitors, 2 μl of each lysate were seeded onto nitrocellulose membrane and blotted with streptavidin. *A*, Bar graph showing percentage of internalized B3 (MesNa-resistant fraction) relative to total protein (membrane-bound and internalized) for five patients. *B*, Representative dot blot of the same experiment. Horizontal lines have been inserted to represent repositioned membrane lanes.

anti-IgM Abs to induce sIg capping in Daudi cells. Results in Fig. 2*D* show that a reduction of sIg expression did not modify B3 binding.

B3 is the most abundant erythrocyte membrane protein and comprises three distinct structural domains: a membrane spanning domain, a short C-terminal cytoplasmic domain and a large N-terminal cytoplasmic domain (B3N) that accounts for half of the protein size. To gain insight into the mechanism of B3 binding to CLL cells, we analyzed the ability of two mAbs directed against the membrane and B3N domains to interfere with B3 recognition. As shown in Fig. 2*E*, B3 binding to CLL cells was not modified by anti-membrane-domain Ab but was completely blocked by anti-B3N Ab, suggesting involvement of the cytoplasmic domain. We confirmed this assumption by obtaining similar binding curves with recombinant B3N (Fig. 2*F*).

Altogether, these results show that soluble B3 binds to CLL cells by a yet uncharacterized mechanism in which the N-terminal region of the erythrocyte protein is recognized in a specific fashion.

CLL cells effectively take up bound B3

We then evaluated whether binding of B3 is followed by its internalization by leukemic CLL cells. To this aim, B3 was biotin-labeled with a compound that contains a disulfide bond in the spacer region. Protein binding could thus be distinguished from endocytosis upon treatment with a membrane-impermeable reducing agent. CLL cells were incubated with B3-SS-biotin in the cold, then washed and finally shifted to 37°C to allow internalization of bound B3. Biotin remaining on the cell surface was cleaved off by

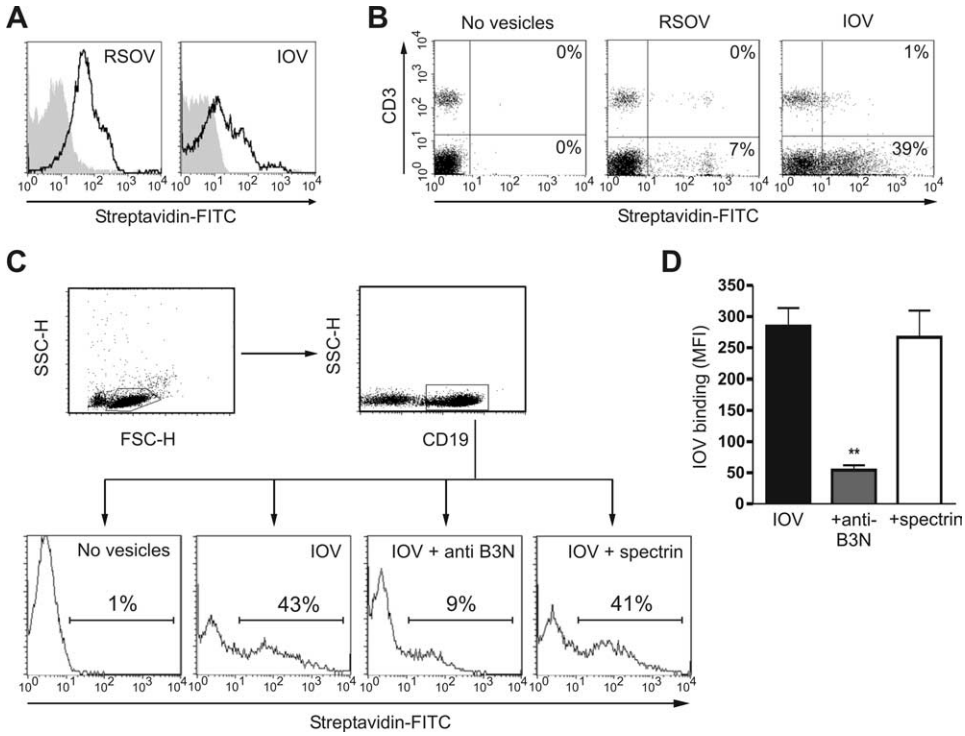

FIGURE 4. CLL cells bind erythrocyte-derived vesicles by interacting with B3. Erythrocyte vesicles were prepared from ghosts under conditions that either favored conservation (RSOV) or reversal (IOV) of the native membrane configuration. For binding experiments, both preparations were tagged with biotin. *A*, Histograms of both vesicle preparations after tagging with streptavidin-FITC. Gray histograms correspond to unlabelled vesicles. *B*, Vesicle binding to leukemic PBMC samples. Cells were incubated with biotin-conjugated vesicles, washed, and then tagged with streptavidin-FITC. Cell populations were discriminated by forward-scattering gating and CD3 labeling, and analyzed by FACS. Dot plots are representative from six samples evaluated. The percentage of cells in right quadrants is indicated. *C*, Effect of anti-B3N Ab on IOV binding to CLL cells. Binding experiments were performed as previously described, but vesicles were blocked with anti-B3 Ab directed to N-terminal domain before adding to leukemic PBMC. Spectrin was used as control protein. CLL cells were discriminated by forward-scattering gating and CD19 labeling. Results are shown as histograms of the $CD19^+$ lymphocyte population (representative patient sample, $n = 6$). *D*, Bar graph shows mean MFI $\pm$ SEM of the six samples analyzed for the three tested conditions: IOV binding alone, in the presence of anti-B3 Ab against the N-terminal domain and spectrin. **, Statistically significant difference ($p < 0.01$) from IOV binding alone.

three rounds of MesNa. As shown in Fig. 3, CLL cells were capable of internalizing bound B3. Total bound protein varied from sample to sample, as it was previously observed by FACS in binding experiments, and so did the fraction of internalized B3. Because cells were first incubated with B3 in the cold and then thoroughly washed before shifting to 37°C, these results cannot be ascribed to fluid-phase endocytosis and the captured protein must have been membrane bound before internalization.

CLL cells bind erythrocyte vesicles by interacting with the N-terminal portion of B3

Because B3 is an integral membrane protein, it seems unlikely that CLL cells in vivo could encounter this protein in a soluble form. Thus, we evaluated whether the leukemic cells were able to bind B3 in its native state within the erythrocyte membrane. To this aim, erythrocyte-derived vesicles with different membrane orientation were prepared: RSOV and IOV. We confirmed that the two different surfaces of the erythrocyte membrane were exposed by using Abs against exofacial and endofacial Ags and FACS analysis. Preparations were >90% correctly oriented (data not shown), in agreement with previous reports (23). Biotin was then conjugated to both RSOV and IOV and comparable levels of labeling were verified by FACS (Fig. 4*A*). Binding of these vesicles was assessed in the same way as for soluble B3, including thorough cell washing before tagging with streptavidin-FITC. As shown in Fig. 4*B*, RSOV showed negligible binding, in contrast to IOV, which bound considerably to CLL cells. To confirm that the same interaction between CLL cells and the N-terminal domain of B3 was involved, we proceeded to evaluate IOV binding in the presence of anti-B3N Ab. As it was the case with soluble B3, under these conditions IOV binding was abrogated, but not when a different erythrocyte-derived protein, spectrin, was present (Fig. 4, *C* and *D*). These findings suggest that the interaction of CLL with B3 is not restricted to its soluble form but that it also occurs in a physiological state of this Ag as erythrocyte-derived vesicles.

T cell responses to B3 with resting and CD40L-activated CLL cells as APC

After the aforementioned binding and endocytosis experiments, we proceeded to evaluate whether CLL cells could indeed drive T cells into proliferation upon presenting B3 peptides. To this aim, we first measured [^{3}H]thymidine incorporation induced by B3 in leukemic PBMC samples previously depleted of monocytes by adherence. In all cases analyzed, the proportion of monocytes was <1% (on average, these cells accounted for 4% of the leukemic PBMC) and there were even fewer normal B cells ($CD19^+CD5^-$). Therefore CLL cells were the only cell type present in sufficient number to act as an Ag-presenting population. Proliferation was assessed on days 5 and 7 after stimulation. Proliferative responses were considered positive when cpm increases greater than mean plus three SD of control wells were observed. Under these conditions we found no positive responses in any of the patient samples examined (Fig. 5*A*). Given that leukemic cells represent in average 90% of total PBMC (ranging from 80 to 95% in the samples analyzed), there were very few T cells in culture. The observed lack of response to Ag could be due to insufficient numbers of responding T cells and not to inability of the CLL cells themselves to process and present B3. Therefore, we proceeded to enrich leukemic PBMC in T cells by partially depleting the samples from $CD19^+$ leukemic cells, thereby increasing T cell fraction to 50% or more and leaving enough CLL cells to act as APC. We assayed proliferation to B3 in these T cell enriched samples (Fig. 5*B*), observing no significant differences between control and Ag-containing cultures.

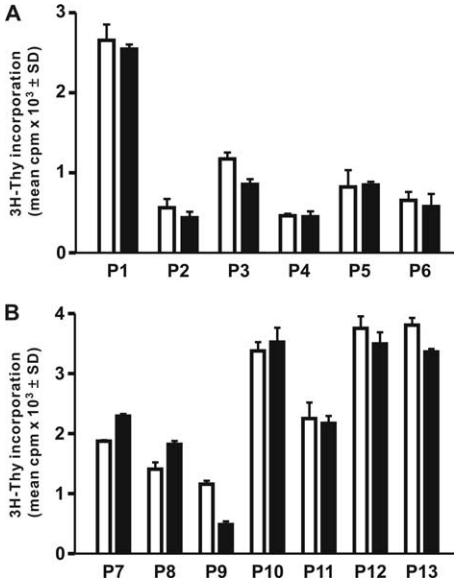

FIGURE 5. Lack of T cell proliferation with resting CLL cells as APC. *A*, Monocyte-depleted PBMC (1.25×10^6 cells/ml) from CLL patients were cultured with medium alone (□) or B3 at a final concentration of 10 μg/ml (■) for 7 days and pulsed with [^{3}H]thymidine for the last 18 h as described in *Materials and Methods*. T cells in these unfractionated samples amounted to 10% total cells (range 5–20%). Bars represent mean cpm ± SD of quadruplicates. *B*, Cultures with monocyte-depleted leukemic PBMC, previously enriched in T cells, either with medium alone (□) or B3 (■). T cell fraction was increased to 50% or more of total cells by depletion with an insufficient amount of anti-CD19 Ab and magnetic beads. Bars represent mean cpm ± SD of quadruplicates.

The inability of CLL cells to present B3 to T cells might be due to the fact that they are poor APC (31, 32). It has previously been shown that CLL cells can be rendered into efficient APC for auto- and alloantigens after engagement of CD40 receptors expressed on their surface (33). This signaling triggers a marked up-regulation of CD80 and CD86 costimulatory molecules, key players in T cell activation whose low expression accounts for the poor APC capacity of resting CLL cells for conventional Ags (34). We hypothesized that this difference would also apply to B3, so we proceeded to purify two cell fractions from PBMC: T cells as responding cells and CLL cells as APC. CLL cells (>99% pure as assessed by FACS) were cultured overnight on CD40L-transfected fibroblasts or untransfected fibroblasts as control. By evaluating CD80 and CD86 expression by FACS, we observed a two- to eightfold increase on CLL cells that were cultured with CD40L-transfected fibroblasts compared with those that were plated over untransfected fibroblasts (Fig. 6*A*). In contrast, no difference in B3 binding capacity was observed between the two cell populations for each of the eleven patients assayed (Fig. 6*B*).

After CD40 ligation, we pulsed CLL cells with B3 instead of leaving the Ag throughout the culture, ensuring that the leukemic cells, and not other contaminating non-T cells in the responding fraction, were acting as APC. By measuring [^{3}H]thymidine incorporation on days 3, 5 and 7, we found that proliferative rates peaked on the fifth day in most cases (data not shown) and thus we selected day 5 as the most representative timepoint (Fig. 6*C*). Activated CLL cells behaved quite differently from their resting counterparts. We obtained positive responses in five of 11 patients analyzed in this way. In all cases responses were blocked by anti HLA-DR Ab, confirming that the proliferation measured corresponded to $CD4^+$ T cells. It is noteworthy that cultures without Ag showed relatively high

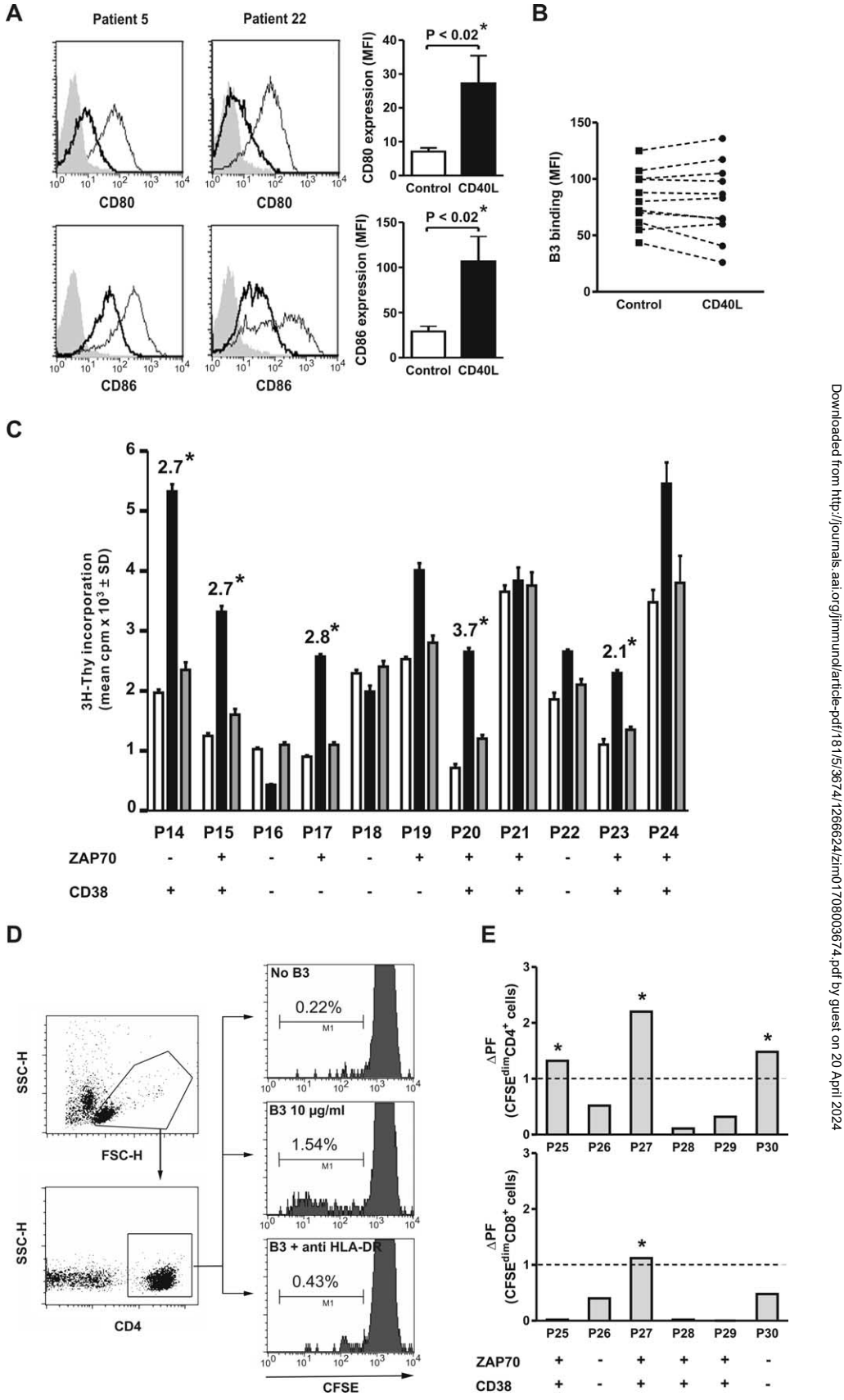

FIGURE 6. CD40L-activated, B3-pulsed CLL cells induce T cell proliferation. *A*, Modulation of B7 costimulatory molecules induced by CD40 engagement. Purified CLL cells (1.25×10^6 cells/ml) were incubated overnight over a monolayer of γ-irradiated CD40L-transfected or untransfected fibroblasts. CD80 and CD86 expression was measured by FACS by gating on $CD19^+$ cells. Histograms from two representative samples are shown. Thick lines correspond to CD80 and CD86 expression in CLL cells incubated over untransfected fibroblasts, thin lines correspond to CD40L-stimulated cells. Gray histograms depict staining with mouse isotype control. Bar graphs show CD80 and CD86 expression in CLL cells of all patient samples (MFI ± SEM, $n = 11$) analyzed. *B*, B3 binding (10 μg/ml) was measured as previously described in both resting (■) and activated (●) CLL cells in the same patient samples ($n = 11$) used for the proliferation assays depicted in Fig. 6*C*. An aliquot of cells was taken after overnight culture over CD40L-transfected or untransfected fibroblasts. *C*, Proliferative responses to B3 with CD40L-activated CLL cells as APC. CLL cells were either pulsed with 10 μg/ml B3 (■), 10 μg/ml B3 plus blocking anti-HLA-DR Ab (▨), or incubated with medium alone (□) as described in *Materials and Methods*. After γ-irradiation, CLL cells (2×10^6/ml) were cocultured with purified T cells (2×10^6/ml) for 5 days and pulsed with [^{3}H]thymidine for the last 18 h. Bar graph shows mean cpm ± SD of quadruplicates, with SI on top for the positive responses, calculated as an increase greater than the mean cpm + 3 SD of control samples. *D*, Proliferative responses to B3 assessed by CFSE dilution assay. CFSE-labeled T cells (10^6/ml) were cocultured for 5 days with CD40L-activated CLL cells (10^6/ml) prepared as described above. Results from a responding patient to B3-pulsed CLL cells are shown. Percentage of $CFSE^{dim}$ cells were calculated on $CD4^+$ viable cells. *E*, $CD4^+$ and $CD8^+$ T cell responses of the six samples evaluated, plotted as Δ proliferation fraction (ΔPF). A response with a ΔPF of at least 1% and a SI of at least 2.0 was considered positive (indicated with ∗). ZAP-70 and CD38 expression for all patient samples analyzed are shown below *C* and *E*.

[^{3}H]thymidine incorporation (cpm >1000), which has been shown to be due to T cell responses against the CLL cells themselves (35, 36).

We confirmed that $CD4^+$ T cells were the responding population to B3-pulsed CLL cells by performing the same experiments in six independent samples, but tagging the T cell fraction with

CFSE. In three of the samples, we found at least a two-fold increase in $CFSE^{dim}CD4^+$ cells that was abrogated in the presence of anti-HLA-DR Abs. Fig. 6*D* shows representative cytometry data from a responding patient to B3-pulsed CLL cells and Fig. 6*E* shows both $CD4^+$ and $CD8^+$ proliferative fractions of the six samples, calculated as the difference between the specific proliferation and the background. With one patient, an increase in $CD8^+$ T cells was also found, but this proliferation reverted in the presence of blocking HLA-DR Ab, suggesting that it was due to bystander proliferation induced by $CD4^+$ T cell activation. Altogether, these data show that CD40L-activated CLL cells that have been pulsed with B3 can induce $CD4^+$ T cell proliferation by a HLA-DR-dependent mechanism, indicating that Ag presentation is involved.

Noteworthy, when CD38 and ZAP70 expression status of the 17 patient samples assayed was considered, only one of five patients with favorable prognosis had a positive response but seven of 11 patients with unfavorable clinical prognosis (at least one positive marker) showed T cell proliferation to B3 when their CLL cells were used as a APC (Fig. 6, *A* and *C*). Although the sample size is too small to achieve statistical significance, this difference between CLL subgroups deserves further investigation.

Discussion

In the present study, we describe for the first time the existence of a binding site for the erythrocyte Ag B3 on CLL cells that enables them to concentrate and take up the protein. We also show that, if appropriately stimulated, B3-pulsed CLL cells are able to induce $CD4^+$ T cell proliferation. From these data, we propose that the malignant cells may trigger the autoaggressive process in AHA secondary to CLL by presenting B3 epitopes to autoreactive T cells.

Although not when in soluble form, B3 binding has been previously reported in the case of *Plasmodium*-infected erythrocytes, where the parasite's metabolism induces oxidation and aggregation of this protein on the cell surface (17). CD36, through recognition of altered B3, is responsible for the adherence of these abnormal red cells to monocytes and more importantly, endothelial cells (18, 28). This type B scavenger receptor is expressed on endothelial cells and monocytes but not on normal B lymphocytes (37, 38). However, variable CD36 expression has been described on CLL cells and its levels correlated to disease stage (19). We considered that this receptor could also be involved in soluble B3 binding, but neither a blocking anti-CD36 Ab nor other competing scavenger receptor ligands could modify total bound protein. Another interaction of B3 with a cell receptor has been described involving the N-glycan. Nucleolin (29), a multifunctional shuttling protein expressed in monocytes and B cells, binds the conformational motif of aggregated B3 glycan that is induced by oxidation and thus has been implicated in senescent erythrocyte turnover. In contrast, B3 binding to CLL cells was not modified after enzymatic deglycosylation, indicating that the glycan is not relevant in this case. Confirming the different nature of this interaction, recombinant N-terminal domain of B3 bound to CLL cells in a concentration-dependent manner. This finding and the fact that a monoclonal anti-B3 Ab specific for the N-terminal region abrogated B3 interaction with CLL cells demonstrate that this is the relevant domain in the phenomenon described. It should be noted that this portion of the erythrocyte anion exchanger is entirely intracytoplasmic and therefore it seems unlikely that CLL cells could interact with intact, circulating red cells. However, erythrocytes spontaneously shed fragments of their membrane (39) and both RSOV and IOV have been demonstrated in blood plasma samples (40, 41). When we assessed binding of both types of vesicles to CLL cells, we found that only IOV markedly bound to leukemic cells. More importantly we observed that this binding was abrogated in the presence of anti-B3N Ab, indicating that the interaction between CLL cells and vesicles relies on this cytoplasmic domain. Given that the accumulation of leukemic cells within the splenic stroma is a feature of CLL and this organ is responsible for the "pitting" or removal of such vesicles (40, 42) as the red cells traverse the splenic sinusoids, it is highly probable that CLL cells could encounter inside-out erythrocyte fragments displaying the N-terminal domain of B3.

CLL cells not only can bind B3, but they are also able to take up bound protein, as evidenced by our data from endocytosis assays. These findings do not imply that the putative receptor has endocytic capacity, because it could simply concentrate B3 on the cell surface, which could later be taken up by other endocytosis mechanisms. In either case, this erythrocyte protein ends up inside the cell, potentially available to proteolytic enzymes. Collectively, these data show that CLL cells can bind and take up B3, two steps necessary for APC function.

B cells do not have an important part in the initiation of the response against infection, but have emerged as key players in the triggering of immunity against self (43–46). In contrast, neoplastic cells from several B lymphomas exhibit an efficient capacity to process and present Ags to T cells (47). By putting these observations together, the possibility of CLL cells acting as APC for AHA Ags seems attractive. Hall et al. (9) have previously reported that these leukemic cells can present Rh proteins to T cells, although it is unclear how they could take up the Rh Ags and present them efficiently as CLL cells are usually regarded to as poor APC even for conventional Ags (31, 32). In agreement with this prevailing notion, we found that circulating CLL cells, despite being able to bind and take up B3, cannot efficiently present it to T cells. However, upon being activated by culture on CD40L-transfected fibroblast, CLL cells pulsed with B3 induced T cell proliferation in eight of 16 patients evaluated by [^{3}H]thymidine incorporation or CFSE dilution assay. It is noteworthy that none of our patient samples had detectable erythrocyte-reactive Abs in serum either by Coombs testing or FACS (data not shown) and yet half of them showed positive proliferative responses to the erythrocyte Ag B3 under the appropriate settings. Although striking at first, our findings are in agreement with previous reports from Barker and Elson in AHA (48) and from different groups in other autoimmune diseases where the prevalences of positive T cell responses in patients and healthy donors were alike (49–53). There were, however, differences in the quality of the responses. For example, autoreactive T cells from patients with type 1 diabetes mellitus or multiple sclerosis differ from those of healthy subjects in their state of activation, being markedly less dependent on costimulation, which suggests that they are activated/memory cells. Moreover, autoreactive T cells in type 1 diabetes mellitus show a polarization toward IFN-γ production, whereas those of healthy controls are polarized toward IL-10 secretion in response to islet peptides. It remains to be determined whether B3 presentation by CLL cells leads to an outcome different from that of classical APC, such as monocytes and dendritic cells.

In a murine model of autoimmune diabetes (54), B cells proved to be crucial in priming autoreactive T cells, but once the process had begun, other APC populations could sustain the immune response. In AHA, this could be the case for the proliferation observed with CLL cells as APC, serving as igniters by presenting certain B3-derived peptides and setting the appropriate inflammatory stage for another APC to carry on the immune response by epitope spreading (46, 55). Because AHA also affects patients without CLL and the autoantibodies involved share the same reactivity profiles (7), the leukemia must be a predisposing, but not

determining factor in the pathogenesis of this autoimmune disease. Spleen enlargement due to leukemic infiltration usually precedes the onset of AHA in the clinical course of CLL, and this increase in size is accompanied by an increase in splenic functions (56, 57). These leukemic cells are at the same time receiving survival signals from surrounding stromal and T cells, one of which is CD40-CD40L interaction (58). In this physiological setting, it is conceivable that activated CLL cells could bind and take up B3, available as a by-product of erythrocyte turnover, and then present it to T cells in the presence of appropriate costimulation. More available Ag (from faster erythrocyte turnover) could lead to higher levels of presentation (eventually reaching the threshold of cryptic epitopes) (59), and/or a different processing machinery in CLL cells could generate novel epitopes to the immune system (60). Either situation or the sum of the two would in turn lead to autoreactive T cell activation and proliferation, a determinant step in any autoimmune process. It remains to be determined which are the dominant epitopes in T cell responses to B3 and if either of the possibilities mentioned above actually contributes to the triggering of AHA in CLL patients. Remarkably, only one of the five patients with favorable clinical prognosis had a positive T cell response whereas seven of 11 patients with unfavorable prognosis showed proliferation to B3. An underlying difference in the Ag-presenting capacity of leukemic cells between the two CLL subgroups could potentially contribute to the increased likelihood of developing AHA observed in aggressive CLL cases.

In conclusion, our research supports the idea that the leukemic clone in CLL could be acting as an Ag-presenting population for B3 in the generation of AHA. The underlying mechanisms involved in this process should be better characterized for their potential as therapeutic targets.

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Disclosures

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