Therapeutic implications of variable expression of CD52 on clonal cytotoxic T cells in CD8+ large granular lymphocyte leukemia

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ABSTRACT

Background

T-cell large granular lymphocytic leukemia is a clonal proliferation of cytotoxic T-lymphocytes which often results in severe cytopenia. Current treatment options favor chronic immunosuppression. Alemtuzumab, a humanized monoclonal antibody against glycophosphatidylinositol-anchored CD52, is approved for patients refractory to therapy in other lymphoid malignancies.

Design and Methods

We retrospectively examined treatment outcomes in 59 patients with CD8+ T-cell large granular lymphocytic leukemia, 41 of whom required therapy. Eight patients with severe refractory cytopenia despite multiple treatment regimens had been treated with subcutaneous alemtuzumab as salvage therapy. Flow cytometry was used to monitor expression of glycophosphatidylinositol-anchored CD52, CD55, and CD59 as well as to characterize T-cell clonal expansions by T-cell receptor variable β-chain (Vβ) repertoire.

Results

Analysis of the effects of alemtuzumab revealed remissions with restoration of platelets in one of one patient, red blood cell transfusion independence in three of five patients and improvement of neutropenia in one of three, resulting in an overall response rate of 50% (4/8 patients). Clonal large granular lymphocytes exhibited decreased CD52 expression post-therapy in patients refractory to treatment. Samples of large granular lymphocytes collected prior to therapy also unexpectedly had a significant proportion of CD52-negative cells while a healthy control population had no such CD52 deficiency (p=0.026).

Conclusions

While alemtuzumab may be highly effective in large granular lymphocytic leukemia, prospective serial monitoring for the presence of CD52-deficient clonal cytotoxic T-lymphocytes should be a component of clinical trials investigating the efficacy of this drug. CD52 deficiency may explain lack of response to alemtuzumab, and such therapy may confer a survival advantage to glycophosphatidylinositol-negative clonal cytotoxic T-lymphocytes.

Key words: large granular lymphocytic, alemtuzumab, therapy.

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Introduction

Large granular lymphocyte (LGL) leukemia is a clonal lymphoproliferation of cytotoxic T-lymphocytes (T-LGL) or natural killer cells (NK-LGL), frequently accompanied by lineage-restricted cytopenias.[1-7] Though often asymptomatic, LGL leukemia may be associated with complications related to cytopenias, most commonly neutropenia. High-dose chemotherapy is usually ineffective, and better hematologic responses may be achieved with chronic low-dose immunosuppressive therapy. Although various medications have been used, inherent toxicities are problematic and some patients remain refractory to treatment. The availability of effective therapy is important to avoid toxic side effects of chronic treatments, neutropenic complications, transfusion dependence, and other associated morbidity.

Typically, T-LGL leukemia is a disease of middle to late adulthood with the median age of patients at presentation being about 55-60 years; males and females are equally affected. The disease course may be indolent and chronic in the majority of cases, and is asymptomatic for long periods in about a third of patients. Neutropenia is the most common hematologic presentation, occurring in over two-thirds of T-LGL patients, and can be accompanied by infections or febrile neutropenia.[4,8-14] However, severe episodes of infections occur less frequently in T-LGL patients, despite low absolute neutrophil counts (ANC), than in patients with neutropenia in the context of typical hematologic malignancies. The extreme monoclonal expansions within the T-cell repertoire typical of T-LGL would suggest impaired antigen clearance, yet opportunistic infections are unusual. Other lineage-restricted cytopenias, including red cell aplasia and thrombocytopenia, occur less frequently than neutropenia.[1,3,9,12,15] Interestingly, in some patients, lineage-restricted cytopenias and/or pancytopenia may occur interchangeably at different times during the course of the disease.[16]

According to one theory, T-LGL arises in the context of a reactive polyclonal cytotoxic T-lymphocyte (CTL) expansion, undergoing transformation in a manner similar to that proposed for chronic lymphocytic leukemia.[17,18] As such, one of the effector CTL clones, at first driven by the presence of its cognate antigen, may transform and thus fail to undergo apoptosis.

Cytopenias could initially be secondary to a T-cell-mediated process in the absence of clonal predominance, i.e. before the occurrence of the subsequent clonal transformation. The presentation spectrum of LGL leukemia is determined by the specificity of the T-cell receptor: if myeloid precursors are targets of clonal CTL, neutropenia will be a clinical manifestation, whereas if erythroid progenitors are affected, anemia will be present.[19] Thus, cytopenias may resolve following immunosuppression, yet the CTL clone may persist at a certain level, suggesting that other disease mechanisms contribute to clonal survival. Various soluble effectors including Fas-ligand have also been implicated in the pathophysiology of the cytopenias in T-LGL.[20-22]

The clonal CTL in T-LGL generally resemble an antigen-activated $CD8^+CD57^+$ effector cell, as both constitutively express perforin and Fas-ligand and can suppress neutrophils *in vitro*.[21,23,24] Gene expression studies have shown that the T-LGL clone exhibits upregulation of cytotoxic proteases and adhesion molecules and downregulation of protease inhibitors.[23]

Alemtuzumab (Campath-1H) is a humanized monoclonal antibody recognizing the CD52 antigen and is capable of selectively killing CD52-expressing cells.[25] CD52 is widely present on most human lymphocytes, expressed on the cell membrane via a glycosylphosphatidylinositol (GPI) anchor. The mechanism of action includes both antibody-dependent cellular cytotoxicity and complement-mediated lysis. Alemtuzumab was initially utilized in the treatment of lymphoid malignancies, in which it showed excellent lymphotoxic effects. Due to its strong anti-lymphocyte action, alemtuzumab has been tested in autoimmune diseases[26-29] and, more recently, in stem cell transplantation.[30-32] In both conditions, use of the drug has resulted in profound lymphocyte depletion, affecting all lymphocyte subsets, yet it should be noted that the intensity of CD52 expression varies among lymphocyte subsets, with highest expression on CD4 and B cells, moderate expression on CD8 cells, and heterogeneous expression on NK cells.[33] Alemtuzumab was approved in 2001 for the treatment of lymphoid malignancies in which alkylating agents and fludarabine therapy had failed. In this study, we analyzed the efficacy of current therapies for $CD8^+$ T-LGL leukemia including alemtuzumab and investigated the mechanism of alemtuzumab resistance.

Design and Methods

Patients

Peripheral blood samples were collected from patients during clinically indicated testing following informed consent, according to the protocols approved by the Institutional Review Board of the Cleveland Clinic. A retrospective chart review was carried out based on the procedures of an Institutional Review Board-approved protocol. The diagnosis of T-LGL required the presence of three of the following criteria: monoclonal Tγ T-cell receptor rearrangement determined by polymerase chain reaction, an LGL count greater than 1.5×10^9/L, flow cytometric evidence of an abnormal CTL population characterized by expression of CD2, CD3, CD4, CD8, CD16/56 or CD57 with negativity of CD28, and the presence of T-cell receptor variable β region (Vβ) expansion according to criteria previously described.[34] Cytopenias were classified as neutropenia (absolute neutrophil count, ANC < 1.5×10^9/L), anemia (hemoglobin <11 g/dL), and thrombocytopenia (platelet count $<100\times10^9$/L). Clinical responses were determined according to the modified International Working Group criteria for myelodysplastic syndromes, as previously reported.[35] The patients' clinical characteristics are displayed in Table 1.

Flow cytometry

Fresh peripheral blood was stained for Vβ flow cytom-

etry analysis to quantitate the percentage of each Vβ family in the CD4 and CD8 lymphocyte populations. The manufacturer's instructions (IOTest Beta Mark kit, Beckman-Coulter, Fullerton, CA, USA) were modified as follows: 5 μL of phycoerythrin cyanin 5-conjugated anti-CD4 (Beckman Coulter), 5 μL of phycoerythrin cyanin 7 anti-CD8 (BD Biosciences) monoclonal antibodies were added. Anti-Vβ 6.7 fluorescein isothiocyanate (FITC) (Pierce Biotechnology, Rockford, IL, USA) was also included in the panel. A four-color acquisition protocol was used on a Beckman Coulter FC500 with CXP software. Either CXP or FCS Express (De Novo Software, Los Angeles, CA, USA) was used for analysis. The lymphocyte gate was set according to forward and side scatter properties. For Vβ family T-cell repertoire analysis, gates were set on CD4 and CD8 bright lymphocyte populations and then analyzed for Vβ distribution. Mean and standard deviation (SD) values were provided by the manufacturer of the IOTest Beta Mark Kit and based on a control population of 85 volunteers, as previously described.[36] The Vβ repertoire in a control group of 37 volunteers was analyzed within our laboratory and results did not differ significantly from those of the Beckman group or previous publications.[37] A significant clonal expansion was defined as one that was greater than the mean plus two SD of healthy controls. The mean and cut-off values from our control population were (average, +2SD; all values are expressed as percentages of either the CD4 or CD8 T cell compartment): CD4 Vβ1 3.2, 5.13; CD4 Vβ2 8.52, 12.84; CD4 Vβ3 4.2, 8.99; CD4 Vβ4 1.45, 2.76; CD4 Vβ5.1 6.25, 9.54; CD4 Vβ5.2 1.03, 1.64; CD4 Vβ5.3 0.95, 1.8; CD4 Vβ7.1 1.69, 3.13; CD4 Vβ7.2 1.28, 2.73; CD4 Vβ8 5.24, 9.57; CD4 Vβ9 2.75, 4.9; CD4 Vβ11 0.87, 2.42; CD4 Vβ12 2.18, 4.02; CD4 Vβ13.1 3.99, 6.46; CD4 Vβ13.2 2.48, 4.35; CD4 Vβ13.6 1.65, 2.53; CD4 Vβ14 2.69, 4.47; CD4 Vβ16 1.16, 2.38; CD4 Vβ17 5.26, 7.96; CD4 Vβ18 1.06, 2.07; CD4 Vβ20 2.94, 5.76; CD4 Vβ21.3 2.58, 5.43; CD4 Vβ22 3.75, 6.34; CD4 Vβ23 0.38, 0.79; CD4 Vβ6.7 4.9, 7.79; CD8 Vβ1 4.49, 9.35; CD8 Vβ2 4.68, 8.48; CD8 Vβ3 3.97, 9.85; CD8 Vβ4 0.83, 2.66; CD8 Vβ5.1 2.2, 4.87; CD8 Vβ5.2 1.44, 4.57; CD8 Vβ5.3 1.0, 1.99; CD8 Vβ7.1 3.19, 6.61; CD8 Vβ7.2 2.52, 5.47; CD8 Vβ8 4.17, 8.0; CD8 Vβ9 2.19, 5.07; CD8 Vβ11 1.09, 4.07; CD8 Vβ12 1.73, 4.61; CD8 Vβ13.1 3.36, 6.76; CD8 Vβ13.2 3.43, 7.3; CD8 Vβ13.6 1.23, 2.7; CD8 Vβ14 4.94, 9.45; CD8 Vβ16 2.51, 9.80; CD8 Vβ17 4.85, 12.43; CD8 Vβ18 0.95, 3.03; CD8 Vβ20 2.43, 6.06; CD8 Vβ21.3 3.21, 7.21; CD8 Vβ22 2.8, 6.81; CD8 Vβ23 1.87, 4.28; CD8 Vβ6.7 2.08, 4.88. In the absence of a detectable CD8 LGL clone by the Vβ panel, indirect evidence of clonality was inferred based on a positive monoclonal Tγ rearrangement test, reversed CD4/CD8 ratio, and the absence of reactivity to antibodies in the Vβ panel, as previously discussed.[37]

Four- or five-color flow cytometry was performed for patients and controls. In brief, within the lymphocyte gate, anti-CD8 phycoerythrin cyanin 7 and anti-CD3 phycoerythrin- or phycoerythrin Texas red (ECD)-(Beckman Coulter) labeled lymphocytes were analyzed for the expression of CD52 allophycocyanin, NKG2D phycoerythrin (Biolegend, San Diego, CA, USA) and CD57 FITC (Beckman Coulter). Using the previously mentioned IOTest Beta Mark kit, comprising 24 Vβ specific antibodies, Vβ restriction of the LGL clone was determined, if not already known through previous research. In some cases, this allowed for the use of the appropriate anti-Vβ antibody in conjunction with anti-CD52 to precisely measure expression of CD52 on LGL cells. In additional experiments, CD52 staining was combined with FITC-labeled markers of GPI-deficiency CD55 and CD59 to assess whether the absence of CD52 also correlated with the lack of other GPI-anchored proteins. At least 10,000 lymphocyte-gated events were collected.

Table 1. Clinical characteristics of patients with large granular lymphocyte leukemia.

Parameter	
Age (years)	62±10
Gender (male/female)	33:26
LGL count (×10⁹/L)	2.934±4.348
Vβ clone size of CD8 cells (%)	63±33
Hematologic manifestation (%)	
Neutropenia	68
Anemia	42
Thrombocytopenia	17
Any	90
Asymptomatic	10

Statistical analysis

To compare CD52 expression between LGL and normal samples, Wilcoxon's rank sums test was used in JMP 7.0 (SAS Institute Inc., Cary, NC, USA).

Results

Patients and therapy results

We reviewed the clinical parameters at presentation and the therapies received in a cohort of patients with T-LGL seen in consultation or treated at the Cleveland Clinic Taussig Cancer Institute from 2002 to 2008 (Table 1). Of these patients, 40 presented with neutropenia, 25 with anemia, and 17 with mixed cytopenias, while six patients were asymptomatic and only mildly cytopenic. We focused our analysis on the therapies received and the clinical responses achieved (Table 2). Of 59 T-LGL patients, 18 did not require therapy. The decision to treat was determined by the following clinical criteria: significant symptomatic anemia (<9 g/dL) and/or the need for transfusion (N=11), severe neutropenia (ANC <0.5×10⁹/L) associated with at least one episode of infectious complications (N=12), severe thrombocytopenia (<50×10⁹/L, N=2) or a combination of these parameters (N=16). Of these patients, 16 received cyclosporine A as primary therapy, five oral cyclophosphamide, four CHOP or intravenous cyclophosphamide, and three oral methotrexate, and these treatments resulted in 7/16, 2/5, 1/4 and 1/3 responses,

respectively, so that no further therapy was needed. Among the patients who did not respond to initial therapy, four received cyclosporine A, seven cyclophosphamide, and three metotrexate as a second-line regimen, with hematologic responses in 3/4, 5/7, and 1/3, respectively. Of the refractory patients, two received antithymocyte globulin and both responded to this treatment. Overall, the response rate to cyclosporine A was 12/27 (44%), to oral cyclophosphamide 7/16 (44%), and to methotrexate 3/8 (38%). Other salvage agents included prednisone and danazol. Splenectomy was performed on 12 patients and was effective in five patients. In our practice, we have encountered eight patients with symptomatic cytopenias who were refractory to multiple regimens and received alemtuzumab as a salvage regimen given either by our institution or by the referring hematologists.

Response to alemtuzumab

We investigated the subsequent clinical course of patients refractory to multiple regimens who, in the course of their management, received therapy with alemtuzumab (Table 3). Four of the eight patients responded to this therapy (1/3 with neutropenia as the primary indication, 3/5 with anemia and 1/1 with thrombocytopenia). Of particular interest, a remarkable response was achieved in a patient with T-LGL who did not respond to multiple regimens and was transfusion-dependent for 3 years, receiving transfusions every 2-3 weeks (Figure 1A). Alemtuzumab was well tolerated and associated with one episode of cytomegalovirus viremia.

When tracked over time, including pre- and post-treatment time points, both relative and absolute lymphocyte counts in responders to alemtuzumab decreased while no significant changes were seen in refractory patients. The mean relative change for responders was –34.7% *vs.* +1.6% for non-responders (Figure 2, left) and the mean absolute change was -5.22×10^9/L in responders *vs.* +0.02×10^9/L in non-responders (*data not shown*). When we measured the size of Vβ clones by flow cytometry, there was a decrease in the size of the LGL clone in three of three responders (mean change -3.362×10^9/L) and one of four non-responders (mean change +0.96×10^9/L) (Figure 2, right). In one illustrative patient, extreme decreases in both relative lymphocyte counts and absolute clone counts were observed.

Table 2. Overall response to standard therapy.

Treatment	Patients who received treatment N. (%)	Patients who responded N. (%)
Cyclosporine A	27 (45)	12 (44)
Methotrexate	8 (14)	3 (38)
Cyclophosphamide	16 (27)	7 (44)
Splenectomy	12 (20)	5 (42)
Rituximab	3 (5)	0 (0)
Antithymocyte globulin	2 (3)	2 (100)
High-dose chemotherapy	6 (10)	1 (17)

Expression of CD52 on normal and large granular lymphocyte clones

We then studied the immunophenotype of patients receiving alemtuzumab in an attempt to explain the refractory cases. Following therapy, we noted that non-responders showed a large proportion of CD52-negative cells, including clonal LGL (Figure 3A), while in responders a reduction in the absolute numbers of clonal cells was observed. A comparison of patients who responded to alemtuzumab and those who failed to do so suggested that there was a higher proportion of non-responders in the LGL CD52-negative group; three of three non-responders whose specimens were available for testing had more than 5% of CD52-negative cells in the CD8 T-cell compartment prior to treatment. As this was not a prospective trial, only two of the alemtuzumab responders had specimens available for testing: one of these two patients had more than 5% CD52-negative CD8 cells. Interestingly, this patient demonstrated an initial response but later relapsed. To assert the hypothesis that down-modulation of CD52 was due to a selection process during therapy, we analyzed archived specimens obtained prior to treatment. A case study of a refractory patient showed a small proportion of clonal Vβ 7.1$^+$ CD52-negative cells pre-treatment (Figure 3A, left plots), which then stained completely negative for CD52 post-treatment (Figure 3A, right plots).

We, therefore, proceeded to analyze a larger cohort of patients who did not receive alemtuzumab and found that a proportion of LGL cases showed decreased expression of CD52 as compared to controls (Figure 1C, N=12 in both groups, p=0.026, Wilcoxon's test). Representative flow cytometric plots of CD52 expression in LGL patients (top) versus control subjects (bottom) are shown in Figure 1D.

We also investigated whether decreased membrane expression of CD52 was isolated or whether other GPI-linked proteins were equally down-modulated. To this purpose we investigated two constitutively expressed GPI-anchored proteins, CD55 and CD59, in a case with down-modulation of CD52 on clonal Vβ 7.1± LGL (82.29% of Vβ 7.1$^+$ CD8 cells were CD52-negative, *data not shown*). We found a large proportion of CD8$^+$CD52$^-$CD55/59$^-$ cells (Figure 3B, top left) while patients with regular CD52 expression displayed the

Table 3. Clinical parameters of patients who received alemtuzumab therapy.

Pt	Presentation	Pre-treatment	Post-treatment	Response
1	Transfusion-dependent anemia	Hb 9.3 g/dL	12.5 g/dL	HI-E
2	Thrombocytopenia	Plt 8×10^9/L	75×10^9/L	HI-P
3	Transfusion-dependent anemia	Hb 9.5 g/d	13.3 g/dL	HI-E
4	Neutropenia Transfusion-dependent anemia	ANC 07.5×10^9/L Hb 7.5 g/dL	1.7×10^9/L 8.5 g/dL	HI-N HI-E
5	Neutropenia	ANC 0.49×10^9/L	0.68×10^9/L	NR
6	Transfusion-dependent anemia	Hb 11.1 g/dL	7.2 g/dL	NR
7	Transfusion-dependent anemia	Hb 11.5 g/dL	10.9 g/dL	NR
8	Neutropenia	ANC 0.8×10^9/L	0.1×10^9/L	NR

Pt: patient; Hb: hemoglobin; Plt: platelets; ANC: absolute neutrophil count; HI-E: hematologic improvement erythroid; HI-P: hematologic improvement platelets; HI-N: hematologic improvement neutrophils; NR: no response.

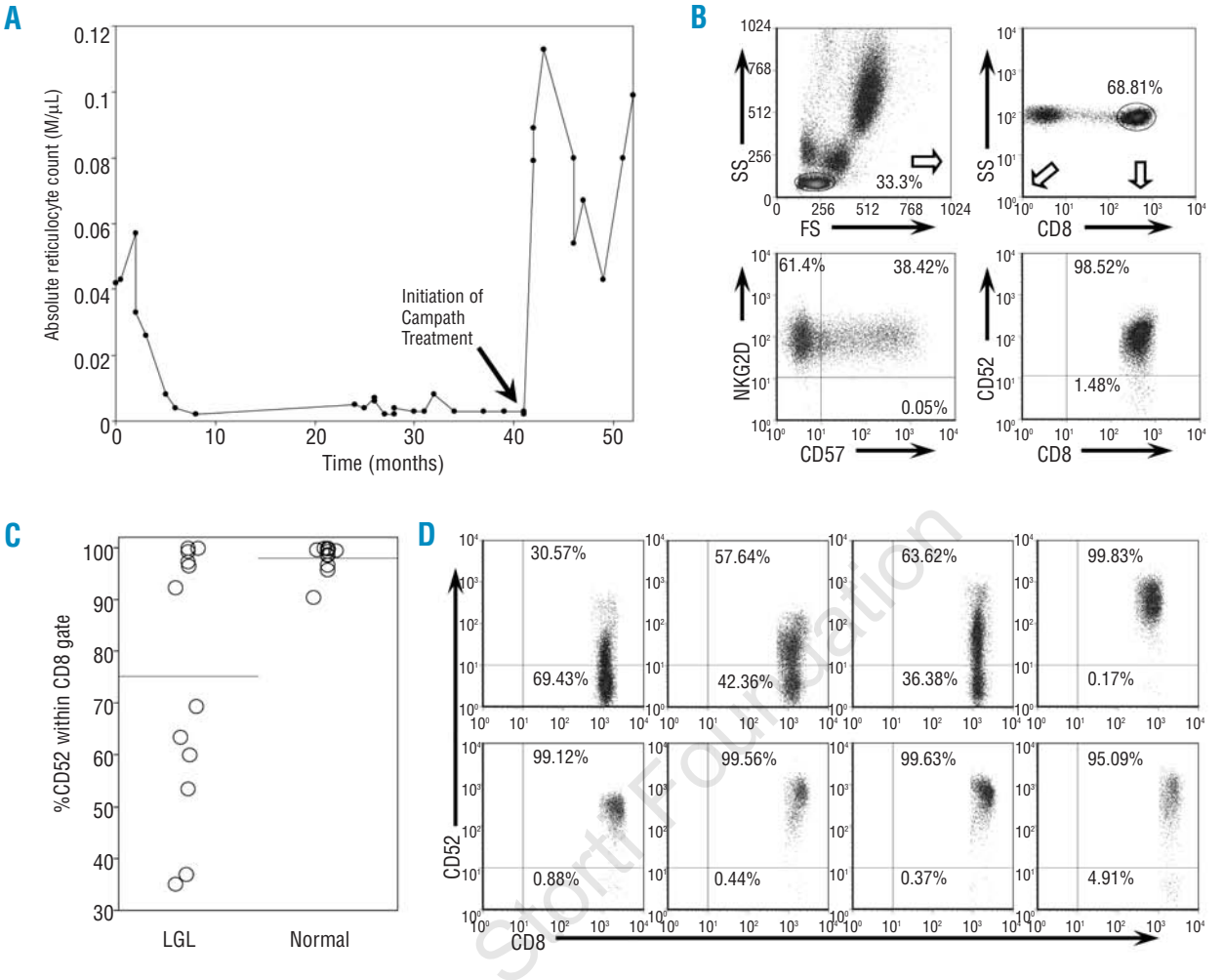

Figure 1. Response to alemtuzumab (Campath) in patients with LGL leukemia may depend on expression of CD52 on CD8 T cells. (A) Exemplary recovery of absolute reticulocyte count in a patient refractory to multiple treatments (early time points) but responsive to alemtuzumab. (B) Gating strategy for flow cytometric analysis of patient in A. This patient's CD8 cells were all positive for NKG2D 38.42% were positive for CD57 and 98.62% were positive for CD52. (C) LGL leukemia patients show significantly reduced levels of CD52 on CD8 cells when compared to healthy volunteers (p=0.026, n=12 in each group, Wilcoxon's rank sums test). (D) Representative flow cytometry results of CD52 expression on CD8 cells. Top panels are data from LGL patients prior to treatment, bottom panels are data from control volunteers.

normal CD55/59 lymphocyte expression pattern (Figure 3B, top center). The deficiency in GPI-anchored proteins was limited to LGL cells, as corresponding granulocytes showed normal expression of CD55 and CD59 (Figure 3B, bottom panels). In contrast, GPI deficiency present in paroxysmal nocturnal hemoglobinuria (PNH) is characterized by a large proportion of $CD15^+$ neutrophils lacking expression of CD59 and CD55.

Discussion

There is no established standard for the therapy of T-LGL-associated cytopenias and, due to a lack of systematic clinical trials, treatment recommendations are mostly derived from case series, case reports, and the empiric application of a variety of drugs used in related con-

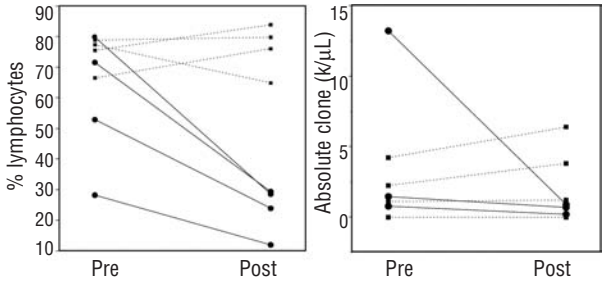

Figure 2. Following alemtuzumab treatment, both the percentage of lymphocytes (left) and the absolute clone counts (right) decreased more in responders (circles, solid lines) than in non-responders (squares, dashed lines). Mean follow-up time was 75±31 days after initiation of therapy. Absolute clone counts were calculated by multiplying the absolute lymphocyte count by the proportion of CD8 cells, then by the proportion of clonal cells as determined by Vβ flow cytometry.

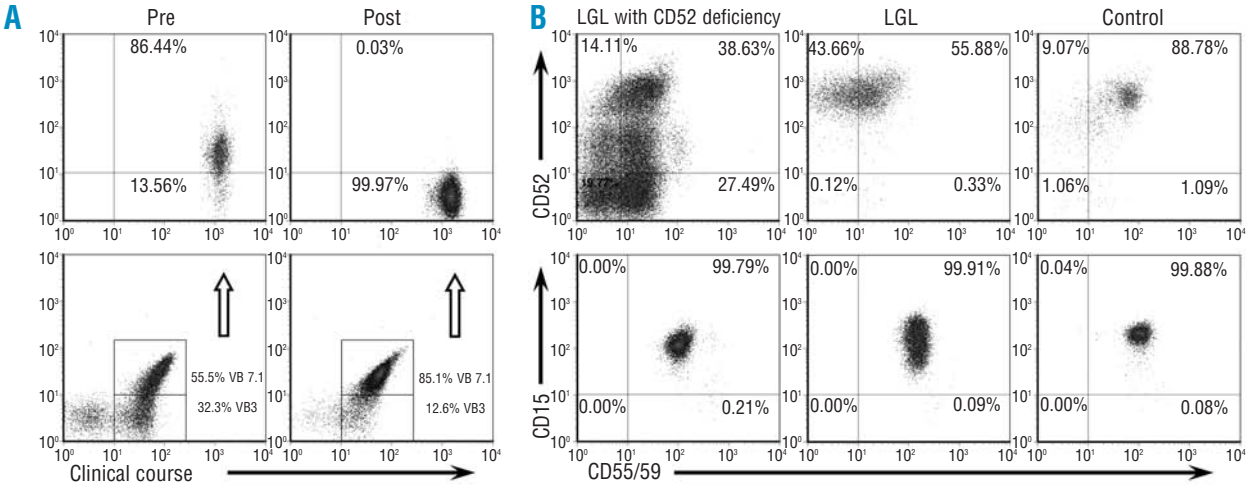

Figure 3. (A) In patients with CD52-deficient CD8 cells, alemtuzumab treatment may result in selection of CD52-deficient cells. Left panels are pretreatment, right panels are post-treatment. Top panels are gated on CD8⁺ Vβ 7.1 as shown below. After alemtuzumab treatment there was a marked increase in the proportion of Vβ 7.1 cells that then stained negative for CD52. (B) CD8-positive, CD52-negative cells from LGL patients were also deficient in other GPI-anchored molecules such as CD55 and CD59. In contrast, neutrophils from LGL patients displayed a normal GPI phenotype. Left panels present data from LGL patients with CD52 deficiency, middle panels present data from LGL patients without CD52 deficiency, and right panels present data from healthy controls. For the upper panels gates were set on lymphocyte and CD8 populations, whereas for the lower panels gates were set on neutrophil populations by forward and side scatter.

ditions. We retrospectively reviewed a cohort of patients evaluated or treated at our institution to assess drug utilization and therapy results in T-LGL. T-LGL can be indolent and at least 10% of patients will remain asymptomatic without need for pharmacologic intervention. Various immunosuppressive therapies are used, mostly to alleviate symptomatic cytopenia; chronic therapy with cyclosporine A, methotrexate or oral cyclophosphamide produces responses in approximately 40% of cases of either neutropenic or anemic patients. The choice of first-line therapy may be dictated by the type of cytopenia present. For example, cyclophosphamide may not be preferred as a first-line therapy in neutropenic patients due to myelotoxicity, and methotrexate is often not used as initial therapy for those with reticulocytopenic anemia. High-dose therapy, including regimens established for B-cell lymphomas, remain largely ineffective and are associated with increased toxicity. Splenectomy appears to be effective in the alleviation of cytopenias. Notwithstanding overall good responsiveness, a proportion of patients with LGL may show a very refractory course despite multiple regimens which may even include high-dose chemotherapy or intense immunosuppression with antithymocyte globulin. In examining options for the further management of these patients, we noted that eight received alemtuzumab during the course of their care, with four displaying hematologic improvement despite prior refractoriness to multiple regimens. Salvage therapy with low doses of subcutaneous alemtuzumab is rational as CD52 is known to be ubiquitously expressed on all lymphocytes and is currently recommended for refractory lymphomas including chronic lymphocytic leukemia. While the clonal T-cell disease is effectively curtailed and immune cytopenias are improved, the treatment does not result in permanent remissions.[38] Most relapses are responsive to repeated cycles of alemtuzumab.

Based on reports of the emergence of lymphocytes deficient in the expression of GPI-anchored proteins mimicking a PNH phenotype after treatment with alemtuzumab,[39-42] we investigated whether down-modulated expression of GPI-anchored CD52 may be responsible for the incomplete response rate documented in our LGL patients. Our observation of CD52-negative LGL in the context of alemtuzumab therapy parallels earlier findings in B-cell lymphoma treated with alemtuzumab: decreased membrane expression of CD52 was accompanied by decreased expression of other otherwise constitutively expressed GPI-deficient proteins, including CD55 and CD59. Previously published data on T-LGL describe lowered expression of CD52 on the cell surface but not complete absence.[43] In contrast to this report and others on B-cell lymphoma,[39-42] we demonstrated here that CD52 is decreased in a subset of patients with LGL as a distinct immunophenotypic feature independent of alemtuzumab therapy. Moreover, we found that patients with CD52-negative LGL are refractory to alemtuzumab treatment and that the proportion of deficient cells increases during therapy, likely due to a selection process. Given the small sample sizes in the alemtuzumab group, these results await confirmation in a larger cohort. Consequently, we conclude that clinical trials of alemtuzumab should include immunophenotypic analysis of CD52 expression. In fact, it is possible that cases of B-cell lymphoma refractory to alemtuzumab may also display down-modulation of CD52 expres-

sion. Indeed, the presence of various fractions of CD52-negative cells has been described in a variety of malignancies, suggesting that CD52 deficiency may be an intrinsic property of various transformed cell types rather than the sole result of alemtuzumab therapy.[44]

In the context of treatment with alemtuzumab, it is possible that due to epitope occupancy of the therapeutic antibody, the antibody we used for flow cytometric detection was prevented from binding. While the observation here that even LGL cells from patients not treated with alemtuzumab display CD52 deficiency underscores the independence of CD52 absence in LGL, it is important that future strategies for the immunophenotypic monitoring of CD52 expression during alemtuzumab therapy account for epitope competition. However, previous research indicates that the murine monoclonal antibody used here (HI186) recognizes an epitope distinct from both the humanized alemtuzumab and the rat clone YTH34.5.[44,45]

The previous observation of the evolution of GPI-deficient lymphocytes in the context of alemtuzumab therapy has been viewed as a presumed immune selection processes analogous to the evolution of GPI-deficient hematopoietic clones in PNH. While this intriguing parallel may be valid, clearly other mechanisms are responsible for the primary down-modulation of GPI-anchored proteins prior to alemtuzumab treatment. One possible explanation would be the acquisition of a *PIG-A* gene mutation, as found in hematopoietic stem cells in PNH. However, GPI-deficient lymphocytes observed after alemtuzumab therapy were shown to contain a wild type *PIG-A* gene.[39,42] In the absence of a mutation, one could argue that the deficient expression of GPI-linked proteins is instead due to down-modulation of genes associated with synthesis of a GPI anchor. Reduced mRNA levels of the *PIG-A* gene have been reported in memory T cells,[46] suggesting that GPI negativity of LGL cells in some patients may be due to a higher proportion of memory cells within the leukemic cell population. It is tempting to speculate that, in both memory and PNH cells, GPI down-modulation is a strategy to avoid immune-mediated cell death. However, $CD8^+CD45RO^+CD62L^{low}$ cells in healthy controls did not display deficiency of GPI proteins CD55/59 or CD52 (*Maciejewski, unpublished observations, 2008*). LGL cells tend not to conform to the classic $CD45RO^+$ memory phenotype, but rather express the CD45RA isoform.[47,48] The implications of this phenotype remain incompletely defined, but functionally these cells are often unresponsive and do not expand *in vitro* using traditional T-cell expansion protocols, suggesting either leukemic transformation by an as yet undisclosed genetic mechanism or functional exhaustion due to chronic antigen stimulation. Furthermore, analysis of CD52 expression in NK-LGL also revealed the absence of GPI proteins on putatively clonal cells (*Maciejewski, unpublished observations, 2008*). We suggest that the mechanisms allowing for the selection of mutant stem cells in PNH and the expansion of T-, NK-, or B-cells with down-modulation of GPI-anchored proteins may have pathophysiological analogies conferring a survival advantage to the GPI-deficient cell.

In summary, we have demonstrated that a primary deficiency of GPI-anchored proteins may be present in LGL and that such a phenotype due to down-modulation of CD52 is consistent with refractoriness to alemtuzumab therapy, which itself can further select this phenotype. Future clinical trials of alemtuzumab in LGL leukemia should prospectively examine the relationship between CD52 expression and response to therapy.

Authorship and Disclosures

JPM was the principal investigator. SRM, MA, NB, and JPM recruited patients, and collected and analyzed clinical data. MJC and JPM designed laboratory experiments. MJC, MWW and HNC performed experiments and analyzed laboratory data. SRM, MJC, MWW, AEL, and JPM designed the study. SRM, MJC, MWW and JPM wrote the manuscript.

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