INTRODUCTION

Induction therapy with alemtuzumab followed by consolidating allogeneic hematopoietic stem cell transplantation in eligible patients is currently considered the standard of care in T-cell prolymphocytic leukemia (T-PLL). Because of its high toxicity, this approach is available only to a minority of the mostly elderly patients and further limited by poor long-term overall survival rates.
of 10%-20%. About 90% of patients respond to alemtuzumab induction, but remissions last for only a median of 12 months. Relapsing disease is usually highly refractory both to conventional chemotherapy and retreatment with alemtuzumab.1,4,5 Molecular mechanisms underlying resistance to alemtuzumab are largely unknown, but may include down-regulation of its target CD52 on the surface of the leukemic cells.6,7 However, the molecular reasons for this immunophenotypic change and its clinical significance remain unclear.

Here, we report a mutational analysis of the GPI-anchor coding PIGA gene in a cohort of 16 patients analyzed by a next generation sequencing (NGS)-based approach. For five patients, pre- and post-treatment samples were available. Two patients presented with CD52-negative T-PLL cells following alemtuzumab treatment. We show that down-regulation of CD52 resulted from loss of GPI-anchor expression caused by inactivating mutations of the PIGA gene. One of the patients achieving a rapid response of the leukemic cells to the anti-CD52 antibody harbored three different PIGA mutations two of which resided in the CD52-negative T-cell population and another in the CD3 negative PBMC fraction. The latter patient developed alemtuzumab-induced paroxysmal nocturnal hemoglobinuria (PNH), which was successfully treated with eculizumab.

2 MATERIALS AND METHODS

2.1 Patients and samples

Patient samples were obtained from fresh or archived cryopreserved material from our institution. Patients were diagnosed in accordance with the WHO 2008 and 2016 classification.8 The study was approved by ethics committee of the University of Duisburg-Essen (14-6080-BO). All patients provided written informed consents according to the declaration of Helsinki. The detection of an inv(14)/t(14;14) or an t(X;14) translocation by cytogenetic analysis and/or detection of TCL1 or MTCP1 breakpoints by fluorescence in situ hybridization (FISH) was required for inclusion into the study. Clinical data of 21 study patients are summarized in Table 1 and Table S1. Standard clinical criteria were applied for initiation of therapy. The patient cohort represents a subgroup of a previously published molecular profiling study investigating the mutational landscape in T-PLL.9

2.2 Tumor cell enrichment and fluorescence-activated cell sorting (FACS)

We performed density gradient centrifugation of heparinized peripheral blood samples (Pancoll human, PAN-biotech). Tumor cells were enriched from peripheral blood mononuclear cells (PBMC) by magnetic cell separation with an anti-CD3 antibody (MACS, Miltenyi Biotech) if the tumor cell content was <90% as detected by flow cytometry. T cells were FACS-sorted into CD52 positive and negative fractions using antibody combinations of CD3 and CD5 and CD52. Details on fluorochromes are listed in Table S2.

Assessment of monoclonality in T cells was performed flow cytometrically with T-cell receptor Vβ repertoire analysis covering approximately 70% of T cells (IO Test Beta Mark, Beckman Coulter). Flow cytometry analysis of PNH was performed with stainings against CD55, CD59, and fluorescein-labeled aerolysin FLAER.10

2.3 Primer design

Primer design for PIGA exon sequencing was performed using the ExonPrimer tool provided by the UCSC Genome Browser webpage and Primer3.11 Primers for the NGS approach were based on the exon structure of PIGA variant 4 (Table 2). The distal part of Exon 2 present in PIGA variant 1, which could not be incorporated into our multiplex strategy, was amplified with primers Exon2_v3.1_F (ACAAACAGTCTAGCCAGCAGC) and Exon2_v3.1_R (AAAGGTTGGGGACAGCATT) and sequenced by conventional Sanger sequencing using Exon2_v3.1_F and Exon2_S1_F (GAGCAGCCTGAATCCTGA) as sequencing primers.

2.4 NGS library preparation

For NGS library sample preparation, two rounds of polymerase chain reaction (PCR) were performed. In the first round PCR, we used gene-specific forward and reverse primers carrying different tags for the second PCR (Table 2). Three and four non-overlapping exon fragments of 242-436 bp in length were amplified in two separate multiplex PCR reactions (QIAGEN Multiplex PCR mix, 40 µL volume) with 25 ng genomic DNA using the following PCR cycling conditions: initial denaturation 2 minutes at 98°C, 35 cycles of denaturation at 98°C for 45 seconds, annealing at 61°C for 45 seconds and extension at 72°C for 45 seconds, followed by a final extension for 5 minutes at 72°C. Reactions were cleaned up with one volume of magnetic
beads (AMPureXP, Beckman Coulter), eluted in 25 µL elution buffer and quality controlled by capillary electrophoresis.

In the second round PCR, the tag sequences were used to introduce specific adapter primers, which contain all elements required for Illumina sequencing and unique dual barcode combinations as identifiers for a specific individual. In our study, these NGS primer pairs consisted of eight different forward and 12 different reverse primers based on Illumina adapters [N/S/E]501-[N/S/E]508 and N701-N712, as described by Leitao et al.12 For this second round of PCR, 1.5 µL of first round product of each multiplex PCR was used

**TABLE 1** Patients' characteristics

| ID  | Sex | Age at diagnosis | CD52 negative T cells (%) | Alemtuzumab treatment | Duration of Alemtuzumab treatment (mo) | Response to treatment | Survival (mo) | PIGA sequencing |
|-----|-----|------------------|---------------------------|-----------------------|----------------------------------------|-----------------------|--------------|----------------|
| 1   | f   | 74               | 0.2                       | no                    |                                        |                       | 34           | x              |
| 2   | f   | 64               | 0                         | no                    |                                        |                       | 18           | x              |
| 3   | m   | 62               | 0.1                       | yes                   | 2                                      | PD                    | 7            |                |
| 4   | m   | 69               | n.a.                      | no                    |                                        |                       | 0            | x              |
| 5   | m   | 75               | 0                         | yes                   | 5                                      | CR                    | 14           | x              |
| 6   | f   | 50               | 0                         | yes                   | 8                                      | CR                    | 28           |                |
| 7   | m   | n.a.             | 0.3                       | no                    |                                        |                       |              | x              |
| 8   | m   | 82               | 0.4                       | yes                   | 3                                      | PD                    | 7            | x              |
| 9   | f   | 62               | n.a.                      | yes                   | 1                                      | PD                    | 21           | x              |
| 10  | f   | 53               | 0.2                       | yes                   | 1                                      | CR                    | 54           |                |
| 11  | f   | 76               | 0.2                       | yes                   | 2                                      | CR                    | 16           | x              |
| 12  | m   | 65               | n.a.                      | yes                   | 5                                      | CR                    | 34           |                |
| 13  | m   | 71               | 0.5                       | yes                   | 5                                      | CR                    | 13           |                |
| 14  | m   | 75               | 0                         | yes                   | 5                                      | PR                    | 32           |                |
| 15  | m   | 70               | 0.2                       | yes                   |                                        |                       | 7            |                |
| 16  | m   | 74               | 0                         | yes                   | 12                                     | PR                    | 7            |                |
| 17  | f   | 74               | n.a.                      | yes                   | 11                                     | CR                    | 29           | x              |
| 18  | m   | 72               | 0.3                       | yes                   | 9                                      | CR                    | 16           | x              |
| 19  | f   | 68               | 1                         | yes                   | 3                                      | CR                    | 24           | x              |
| 20  | m   | 73               | 0.7                       | yes                   | 0                                      | PD                    | 1            |                |
| 21  | f   | 77               | 0.6                       | yes                   | 2                                      | PR                    | 9            | x              |

Abbreviations: CR, complete remission; f, female; m, male; n.a., not assessed; PD, progressive disease; PR, partial remission.

**TABLE 2** Primer sequences used for the NGS approach

| Name               | Multiplex pool # | Sequence 5′>3′                                     |
|--------------------|------------------|---------------------------------------------------|
| Exon1_F            | 1                | ctgctctgtgcacagagGACCTCAGCGCTCTTTTG               |
| Exon1_R            | 1                | caggaaacagctagacAGAAGACCCCATCGGAAGAG              |
| Exon2.1_F_v2       | 1                | ctgctctgtgcacagagGAAATGTGTTTTTGTCTGAGCTG          |
| Exon2.1_R_v3       | 1                | caggaaacagctagacAATAGACTTTTGAGGCCACTG            |
| Exon5_F            | 1                | ctgctctgtgcacagagCCTGAGGTATGATGATGAGGGT          |
| Exon5_R            | 1                | caggaaacagctagacGCAAATGTGACGTGAAACATCAAG          |
| Exon2.2_F          | 2                | cttgctctgtgcacagagGCCCTGATTGGAAAGAGGGCAT         |
| Exon2.2_R          | 2                | caggaaacagctagacTGGAAGAGAGCAGCATGGGCC            |
| Exon3_F            | 2                | cttgctctgtgcacagagTAATGGAAGAGCATGCGG             |
| Exon3_R            | 2                | caggaaacagctagacAAGCAATGACGTAATCCAG              |
| Exon4_F            | 2                | cttgctctgtgcacagagGCCCTGATTGGAAAGAGGGCAT         |
| Exon4_R            | 2                | caggaaacagctagacGCCCTGATTGGAAAGAGGGCAT           |
| Exon6_F            | 2                | cttgctctgtgcacagagGACAGGTGATGGGGTGATG            |
| Exon6_R            | 2                | caggaaacagctagacCCCCAAAAGCAGAGGTATTTTCCA         |
in separate reactions and amplified as following: initial denaturation at 95°C for 10 minutes, 20 cycles of denaturation at 95°C for 30 seconds, annealing at 61°C for 30 seconds and extension at 72°C for 30 seconds, followed by a final extension for 5 minutes at 72°C. The PCR products were again purified with one volume of magnetic beads, analyzed by capillary electrophoresis, quantified spectrophotometrically, pooled and re-quantified by qPCR (NEBNext Library quant kit, New England Biolabs).

### 2.5 | Illumina sequencing and data analysis

Sequencing of the pooled libraries (35 in run1, 3 in run2) was performed on an Illumina MiSeq sequencer using a 600 cycle kit (301/8/B/301 cycles for read1, index 1, index, 2, read2) with a PhiX spike-in of 20%. Demultiplexing was done using the MiSeq reporter software. Generated fastq files were aligned to human genome 19 with BWA mem,13 converted to BAM and sorted with Samtools14 view and sort scripts, respectively. Coverage for all fragments exceeded 1000-fold. Sorted BAM files were imported into StrandNGS (Strand Genomics Inc), quality filtered, locally realigned, and exported. Variant analysis was performed in PartekGS (PartekInc. Partek® Genomics Suite® Revision 5.0, Partek Inc. St. Louis, 2016) using default settings. Called variants were individually inspected in the Integrated Genome Browser.15 Variants were called, if the variant allele frequency was ≥5%.

SEQ files obtained by analysis of Sanger sequences were aligned to the wild-type sequence of PIGA using ClustalW.16 Mismatched positions were individually inspected in the chromatograms using Chromas (Technelysium Ltd) and scored as true, if both of the two sequencing reactions performed with different primers gave consistent results.

### 2.6 | Quantitative reverse transcription PCR

RNA was transcribed into cDNA with the high capacity cDNA reverse transcription kit (Applied Biosystems). Each diluted reverse transcription reaction (1:20) was combined with TaqMan® Universal PCR Master Mix, no AmpErase® UNG (Thermo Fisher) and TaqMan probes for SAMHD1 (Hs00210019_m1) and GAPDH (Hs02758991) (Thermo Fisher). Real-time PCR was performed using AriaMX (Agilent genomics).

### 3 | RESULTS

#### 3.1 | CD52 expression analysis in T-PLL cells

Patient selection for this retrospective study was based on the availability of fresh or viably frozen peripheral blood samples from our T-PLL biobank. The clinical and laboratory characteristics of the majority of patients shown in Table 1 have been previously reported.9 CD52 was found to be homogenously expressed on leukemic cells collected from treatment-naïve patients as determined by multicolor flow cytometry by gating on CD3+ or CD5+ PB lymphocytes. The percentage of leukemic CD52-negative cells was low in this series accounting for a median of 0.28 ± 0.29% (SD, range 0-1) circulating T cells (Table 1).

By contrast, in 2/5 patients (40%) where sequential PB samples were available for longitudinal testing (Table 1) large fractions of CD52-negative T cells could be detected by flow cytometry (Figure 1A,C). Both patients had been initially successfully treated with alemtuzumab. Pretreatment analysis in patient 18 revealed strong CD52 surface expression on the leukemic cells (Figure 1B). Unfortunately, due to the lack of pretreatment material suitable for flow cytometry in patient 12 CD52 expression analysis could only be performed at the mRNA level. Quantitative real-time PCR (qPCR) revealed that CD52 was highly expressed in this patient’s T-PLL cells before the initiation of alemtuzumab treatment as compared to three other T-PLL samples judged to be positive for CD52 by flow cytometry (Table S3).

These data, although limited by the small size of the cohort, show that lack of CD52 surface expression appears to be rare in treatment-naïve T-PLL patients, but may be a rather common phenomenon after treatment with alemtuzumab.

#### 3.2 | CD52-negative T cells lack expression of the GPI-anchor

We next aimed to unravel the mechanism underlying loss of CD52 surface expression in the two index cases 12 and 18. As CD52 expression depends on the expression of an intact GPI-anchor in the cell membrane,17 we employed direct FLAER staining to examine the GPI-anchor in our CD52-negative samples. Figure 2A shows that loss of CD52 expression in case 12 coincided with the absence of the GPI-anchor in 83% of the lymphocytes, whereas accessory non-leukemic granulocytic and monocytic cells in the same sample exhibited a normal aerolysin staining pattern. Comparing pre- and post-treatment samples in this patient using flow cytometry-based Vβ spec- tratyping we confirmed that the CD52-negative T cells exhibited the disease-specific Vβ16-positive TCR clonotype (Figure 3A). Thus, lack of CD52 expression in this patient could indeed be attributed to a GPI-anchor defect in the leukemic cell clone.

Similarly, 86% of PB lymphocytes in case 18 obtained ~4 months after the last infusion of alemtuzumab also showed a GPI-anchor defect. However, different from case 12, loss of GPI-anchor expression was also detected in monocytic and granulocytic cells (Figure 2B). Additional analyses revealed that this also held true for this patient’s red blood cells, 14.7% of which exhibited a CD59 deficient phenotype as detected by flow cytometry. Of note, the TCR clonotype of the CD52-negative T cells differed from the pretreatment sample in that the leukemic cells at diagnosis expressed Vβ17, whereas the CD52-negative T cells stained positive for Vβ12 (Figure 3B). In line with these data, FISH analysis...
of these cells showed the absence of the initially described cytogenetic aberrations including rearrangement of TCRA/D on chromosome 14q11.2 and amplification of MYC residing on 8q24.21. In aggregate, these findings showing loss of the GPI-anchor in multiple hematopoietic lineages are reminiscent of the situation in paroxysmal nocturnal hemoglobinuria. Indeed, patient 18 developed severe transfusion-dependent anemia and the diagnosis of alemtuzumab-induced PNH was established.

3.3 | Loss of GPI-anchor expression is caused by mutations in the PIGA gene

Loss of GPI expression in T cells following treatment with alemtuzumab has been reported to result from mutations in a gene designated phosphatidylinositol glycan anchor biosynthesis class A (PIGA), which plays an important role in GPI-anchor biosynthesis. We next analyzed whether this could also be the case in our two index patients with CD52-/GPI-negative T cells. To this end, we performed targeted NGS of PIGA in our T-PLL samples (Table S4). Whenever technically possible, DNA was extracted from flow-sorted CD52-positive vs CD52-negative T cells, to facilitate intraindividual comparisons of the mutant allele frequencies and enrich for potential PIGA mutations in the CD52-negative cell fraction. In line with our hypothesis, we observed a single protein-damaging mutation affecting exon 3 of the PIGA gene in patient 12 with a higher mutational load in the CD52-negative, as compared to the CD52-positive T-cell fraction (Table 3).

Interestingly, the sample collected from patient 18 with alemtuzumab-induced PNH exhibited a more complex pattern with two
mutations primarily affecting the T-cell compartment and a further mutation in exon 4, which could be exclusively detected in the CD3-negative PBMC fraction (Table 3).

Because treatment with alemtuzumab may lead to the expansion of pre-existing GPI-anchor-deficient CD52-negative cells, we systematically sequenced the PIGA gene in 16 patients before the initiation of treatment. Perhaps unexpectedly, these experiments revealed wild-type sequences in all of the samples tested (Table S4), including the DNA isolated from the two index cases before the initiation of antibody therapy by applying a variant calling in ≥5% of reads.

3.4 | Clinical course and treatment of index patients with CD52-negative T cells

Patient 12, a 68-year-old woman with a high burden of co-existing conditions including chronic hepatitis C virus-induced Child-Pugh B liver cirrhosis, coronary heart disease with atrial fibrillation and diabetes mellitus type 2 relapsed 25 months after first-line treatment with alemtuzumab. The patient presented in poor general condition (ECOG-2) and required immediate cytoreductive treatment because of transfusion-dependent anemia and thrombocytopenia caused by rapid progression of T-PLL. Since flow cytometry demonstrated lack of CD52 expression on the surface of the leukemic cells, combined treatment with ibrutinib and venetoclax was initiated according to a recently published protocol. Treatment was initially well tolerated without evidence of tumor lysis and effective as indicated by a rapid decline of T-PLL cells in the peripheral blood within the first 72 hours of treatment (Figure 4A). Ibrutinib was discontinued on day 35 because of painful inflammatory skin eruptions predominantly affecting both hands. Unfortunately, due to progressive liver failure with onset of hepatic encephalopathy venetoclax also had to be stopped on day 39. The patient was readmitted to the ward and died of progressive hepatorenal syndrome judged to be unrelated to venetoclax treatment.

Patient 18, a 73-year-old man presented with erythrodermia due to leukemic infiltration of the skin, progressive lymphocytosis, and mild lymphadenopathy. FISH was positive for a TCL1A translocation and revealed a chromosomal gain of 8q24.21 (MYC, Table S1). Initially, treatment with alemtuzumab was well tolerated and partially effective as indicated by the rapid decline of the leukemic cells in the peripheral blood (Figure 4B). Alemtuzumab treatment was terminated due to cytopenias after 4 months. However, leukemia cutis resolved only after adding ibrutinib and venetoclax to the treatment schedule. Treatment with ibrutinib/ venetoclax was
discontinued after 1 month due to the development of transfusion-dependent anemia and thrombocytopenia. At this point, flow cytometric and cytologic evaluation showed complete clearance of T-PLL cells from the PB and BM. As detailed in the previous section, Coombs-negative hemolytic anemia could be attributed to the onset of alemtuzumab-induced PNH. Eculizumab treatment was initiated according to a standard protocol resulting in remission of hemolytic anemia (Figure 4B). Eighteen months after the initiation of alemtuzumab therapy the patient is currently in complete remission of his T-PLL. Serial flow cytometric analyses demonstrated a gradual decline of the PNH clone and increasing numbers of FLAER-positive T cells, monocytes, and granulocytes in the PB with continuing follow-up (Figure C).

4 | DISCUSSION

Here, we describe two T-PLL patients initially successfully treated with alemtuzumab in whom peripheral blood cells exhibited a phenotypic switch with loss of CD52 surface expression. There are only few reports on the phenotypic transformation of CD52-positive to CD52-negative T-PLL cells in the literature, where this phenomenon has mainly been discussed as a mechanism for resistance to alemtuzumab. Alemztumab has been associated with the development of CD52-negative blood cells in the setting of allogeneic hematopoietic stem cell transplantation, B-cell non-Hodgkin lymphomas, rheumatoid arthritis, and relapsed chemotherapy-refractory chronic lymphocytic leukemia (CLL). Loeff et al recently showed in a cohort of recipients of alemtuzumab-based T-cell–depleted stem cell grafts that the majority of patients developed CD52-negative donor-derived T cells, which circulated in the PB for years after transplantation in some of the cases. Along the same line, Rawstron et al reported a small CLL cohort, in which 10/15 patients exhibited CD52-deficient T cells after therapy with alemtuzumab. Our data are consistent with these observations in that we could show that CD52-negative T cells emerged in 40% of our alemtuzumab-treated patients, although this needs to be interpreted with caution due to the small number of patients investigated in our study.

Interestingly, despite the fact that CD52 is expressed on all mature lymphocyte subtypes, loss of CD52 in response to alemtuzumab appears to be restricted to the T-cell compartment and rarely occurs in B- or NK-cell. While this phenomenon has been known for some time, the underlying molecular mechanisms have only recently been elucidated. In their transplantation study, Loeff et al showed that loss of CD52 on T cells resulted from loss of GPI-anchor expression caused by inactivating mutations in PIGA. PIGA encodes a protein that is an essential component of an enzyme complex catalyzing the first step of GPI-anchor biosynthesis. As PIGA is located on the X chromosome, a single mutation results in a GPI-anchor-deficient phenotype, because males only have one X chromosome and in females one of the two X chromosomes is inactivated. The detection of PIGA mutations in a small proportion of CD52-negative T cells and
 granulocytes from healthy individuals including hematopoietic stem cell (HSC) donors and alemtuzumab-naïve CLL patients suggests that these cells expand after the onset of selective pressure on CD52-positive cells exerted by alemtuzumab.

By detecting PIGA mutations in both of our patients with a CD52-/GPI-anchor-deficient phenotype, we could show that a similar mechanism is operative in T-PLL. As expected, we observed a higher mutational load in the CD52-negative, as compared to the CD52-positive T-cell fractions. However, different from the work of Loeff et al PIGA mutations could not be detected in the pretreatment samples of our index patients which may be explained at least in part by technical issues. While Loeff et al used a highly sensitive method with sequencing of T-cell clones expanded from FACS-purified single CD52-negative/GPI-negative cells, our sequencing experiments were performed on bulk CD52-negative T cells.

To further investigate the potential presence of PIGA mutations in T-PLL cells collected before the initiation of treatment, we systematically sequenced the PIGA gene in the whole cohort demonstrating a PIGA wild-type configuration in all of the patients. These results are in line with a recent study which also failed to detect PIGA mutations in a series of 54 T-PLL patients analyzed by whole exome sequencing. Along the same line, Kiel et al investigated a cohort of 36 T-PLL patients employing whole exome sequencing, showing a PIGA wild-type configuration in all of the treatment-naive (n = 26) patients. Of note 2/10 (20%) pretreated patients harbored PIGA mutations in their leukemic cell clones, which is consistent with our observation, that CD52-negative/GPI-negative T cells may only emerge after treatment-mediated selective pressure on CD52-positive T cells. Unfortunately, the authors did not reveal the nature of the treatment preceding the collection of T-PLL cells.

On the basis of these results, we cannot exclude a single cell or a small cell clone undetected by NGS giving rise to the CD52-negative T cells in the two index patients originated from PIGA-mutant cells present before the onset of treatment. Thus, we cannot determine whether these mutations have been spontaneously acquired during cell divisions after the initiation of alemtuzumab therapy.

Despite the fact that PIGA gene mutations could be identified as a common mechanism of CD52-negative/GPI-anchor-negative pheno

typic transformation in both of our index patients these two cases differed in some important aspects. In patient 12 suffering from relapsed T-PLL, the CD52-negative/GPI-anchor-deficient phenotype was restricted to the leukemic T-cell clone resulting from a single inactivating mutation in exon 3 of the PIGA gene. Consequently, flow cytometric evaluation of this patient’s granulocytes revealed a normal GPI-anchor expression. By contrast, the CD52-negative/GPI-anchor-negative phenotype in patient 18 affected multiple hematopoietic lineages including non-leukemic T cells, monocytes, granulocytes, and erythrocytes while this patient was in alemtuzumab-induced remission of his disease. Furthermore, this patient’s PB cells harbored mutually exclusive PIGA mutations in the
non-leukemic T cells and in the CD3-negative PBMC fraction. In aggregate, these latter findings suggest that treatment of alemtuzumab resulted in an oligoclonal mutational landscape in the PIGA gene affecting independently the non-leukemic T-cell and hematopoietic progenitor compartments.

(Oligo)clonal expansion of GPI-anchor-negative hematopoietic cells was responsible for the clinical manifestations of the CD52-negative phenotype in both index patients. However, it is now generally accepted that loss of function mutations in PIGA is not sufficient to promote the expansion the affected cells. At least two distinct additional mechanisms of clonal expansion have been demonstrated in PNH cells: i) clonal selection by extrinsic factors, that is, autoimmunity directed at hematopoietic stem cells in the context of bone marrow failure and ii) intrinsic clonal evolution driven by genetic and epigenetic alterations. It is tempting to speculate that both mechanisms acted in combination impinging on the pathogenesis of relapsing disease in patient 12. Alemtuzumab likely initially induced clonal selection of a PIGA-mutant CD52-negative leukemic subclone, which subsequently steadily expanded driven by intrinsic mutations including alterations of TCL1A, MYC, ATM, and TP53 (Table S1), eventually resulting in clinically overt relapse 25 months after the last infusion of alemtuzumab. Conversely, in patient 18 the number of GPI-anchor-deficient cells decreased with time after discontinuation of anti-CD52 antibody treatment (Figure 2B,C), which may suggest that alemtuzumab-mediated immunologic pressure against CD52-positive HSCs alone was responsible for the expansion of GPI-anchor-deficient HSCs to numbers sufficient to cause clinically symptomatic PNH.

Of note, alemtuzumab-induced loss of CD52/GPI-anchors had important albeit different clinical implications in both of the index patients. As reports from the literature indicated that down-regulation of CD52 counteracts the anti-leukemic effects of anti-CD52 antibodies, we refrained from retreating patient 12 with alemtuzumab. Following a recently published investigational protocol, combination therapy with ibrutinib and venetoclax was initiated resulting in rapid decline of T-PLL cells in the peripheral blood within the first 72 hours of treatment. This latter observation is clinically important, since until now efficacy data regarding combined application of ibrutinib and venetoclax in T-PLL patients are scarce.

The clinical picture in patient 18 was dominated by severe transfusion-dependent anemia, resulting from alemtuzumab-induced PNH. To our knowledge, this is the first observation of symptomatic PNH following treatment with alemtuzumab in T-PLL. A search of the literature revealed only one case report describing clinically overt PNH in a patient receiving alemtuzumab as an immunosuppressive therapy of myelodysplastic syndrome. Serial flow cytometric analyses demonstrated a gradual decline of the PNH clone(s) during 12 months of follow-up after discontinuation of alemtuzumab. The kinetics of GPI-deficient T cells in this patient were largely similar to the persistence pattern of PNH-like T cells after allogeneic stem cell transplants utilizing CAMPATH-1H for T-cell depletion. Our patient was successfully treated with a standard regimen of eculizumab and remains currently in treatment-free remission of his T-PLL.

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AUTHOR CONTRIBUTIONS
PJ and JD designed the study, interpreted the data, and wrote the manuscript. LKH performed the sequencing and processed the sequencing data. AR interpreted data. MM performed cell enrichment and cell sorting. HCR and UD wrote the manuscript. All authors read, edited, and approved the manuscript.

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AUTHOR CONTRIBUTIONS
PJ and JD designed the study, interpreted the data, and wrote the manuscript. LKH performed the sequencing and processed the sequencing data. AR interpreted data. MM performed cell enrichment and cell sorting. HCR and UD wrote the manuscript. All authors read, edited, and approved the manuscript.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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