**CD19 Isoforms Enabling Resistance to CART-19 Immunotherapy Are Expressed in B-ALL Patients at Initial Diagnosis**

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**Summary:** B-cell acute lymphoblastic leukemia (B-ALL) is the commonest childhood cancer and the prognosis of children with relapsed or therapy refractory disease remains a challenge. Treatment with chimeric antigen receptor-modified T cells targeting the CD19 antigen (CART-19 therapy) has been presented as a promising approach toward improving the outcome of relapsed or refractory disease. However, 10%–20% of the patients suffer another relapse. Epitope-loss under therapy pressure has been suggested as a mechanism of tumor cells to escape the recognition from CART-19 therapy. In this work, we analyzed the expression of CD19 isoforms in a cohort of 14 children with CD19⁺ B-ALL and 6 nonleukemia donors. We showed that an alternatively spliced CD19 mRNA isoform lacking exon 2, and therefore the CART-19 epitope, but not isoforms lacking the transmembrane and cytosolic domains are expressed in leukemia blasts at diagnosis in children and in the bone marrow of nonleukemia donors. Furthermore, we clarified the sequence of a further isoform lacking the epitope recognized by CART-19 therapy and disclosed the presence of new isoforms. In comparison with the children, we showed that alternatively spliced CD19 mRNA isoforms affecting exon 2 are also expressed in 6 adult patients with CD19⁺ B-ALL. On top of that, one of the adults expressed an isoform lacking the CD19 transmembrane and cytosolic domains. In conclusion, we proved that some of the CD19 isoforms contributing to CART-19 escape already preexist at diagnosis and could evolve as a dominant clone during CART-19 therapy suggesting the application of combined treatment approaches.

**Key Words:** B-ALL, CART-19, epitope-loss, isoforms, CD19

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pediatric and adult B-ALL.16 About 30%–50% of these relapses are characterized by the loss of detectable CD19.15

This raises the question about alternative mechanisms tumor cells use to escape treatment. Epitope loss has been suggested making leukemia cells invisible to the modified T cells. This mechanism is based on the selection of CD19 isoforms lacking either exon 2 and therefore the CD19 epitope FMC63, or the transmembrane and the cytosolic domains resulting in a soluble CD19 variant.17 The existence of such isoforms has only been investigated after anti-CD19-specific therapy so far.13 Therefore, it is not known if these isoforms generally exist before any kind of therapy. Here, we analyzed bone marrow as well as peripheral blood samples of children diagnosed with a CD19+− B-ALL and bone marrow samples of adults diagnosed with a CD19+− B-ALL. All in all, our work suggests that some of the CD19 isoforms responsible for relapse under CART-19 therapy naturally exist before treatment in both children and adults and therefore do not primarily develop under therapy.

MATERIALS AND METHODS

Cohort

All pediatric B-ALL patients, whose samples were investigated, were included and treated as defined in the COALL-08-09 protocol. The control group included children that were hospitalized due to a leukemia suspicion that was not confirmed. Altogether, 14 samples of 13 different children with CD19+− B-ALL as well as a control group of 6 children were analyzed. Six adult B-ALL patients, whose samples were investigated, were treated according to the German Multicenter ALL Study Group protocol.18 Bone marrow and/or peripheral blood samples were obtained as surplus material during standard diagnostic and then frozen in liquid nitrogen. Sample analysis was performed in agreement with the declaration of Helsinki on the use of human material for research. In accordance with the ethics committee of Rhineland-Palatinate, patient’s parents or adult patients agreed with the scientific use of the surplus material and no further approval of the medical ethics committee was required as the data were analyzed anonymously.

Flow Cytometric Immunophenotyping

Flow cytometric immunophenotyping was performed with 100 μL of bone marrow aspirate or peripheral blood by using SSC/CD45 gating. The flow cytometry screen included several lymphoid and myeloid markers, including HLA-DR, CD56, CD16, CD5, CD33, CD13, CD117, CD34, CD19, CD3, and CD45 (all Beckman Coulter, Brea). After staining the samples with these antibodies, red blood cell lysis (8.29 g NH4Cl, 1 g KHCO3, 0.037 g ethylenediaminetetraacetic with 1000 mL Aqua dest) was performed and samples were washed twice with 1× phosphate-buffered saline (PBS) (Sigma-Aldrich, Taufkirchen, Germany). Cells were resuspended in fluorescence-activated cell sorting (FACS) buffer (containing PBS and bovine serum albumin) and measured by a Flow cytometer (NAVIOS; Beckman Coulter). If the leukemic blast population expressed more than 20% of an antigen, it was considered positive. Stored data were analyzed by Navios software (Beckman Coulter).

Fluorescence-activated cell sorting (FACS)

After thawing and counting the cells from bone marrow samples, cells were stained with 7-aminoactinomycin D (7-AAD) viability dye, anti-CD45, and anti-CD19 (all Beckman Coulter) and incubated at room temperature for 15 minutes. Samples were washed once with 1× PBS (Sigma-Aldrich) and the pellet was resuspended in 500–1000 μL FACS buffer (1× PBS with 2 mM ethylenediaminetetraacetic and 2% fetal calf serum), depending on previously counted cells. FACS sorting was performed with FACS Aria (Becton Dickinson, Heidelberg, Germany). 7-AAD+/CD19+/CD45low cells were taken for further analysis.

RNA Extraction and cDNA Synthesis

Total RNA was isolated from bone marrow and blood samples using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. RNA concentration was measured by Nanodrop 2000 (Thermo Scientific, Dreieich, Germany). Quality control was performed by the RNA 6000 Nano Assay by the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) following the manufacturer’s instructions.

cDNA was prepared by using the TaKaRa Prime Script RT reagent Kit with gDNA Eraser (Takara Bio Europe, Saint-Germain-en-Laye, France) following the manufacturer’s instructions. Owing to low RNA concentration after cell sorting, the protocol for cDNA synthesis was changed for sorted samples. Instead of utilizing the RT Primer Mix that is included in the kit, we used self-designed reverse primer specifically amplifying the CD19 gene as well as the housekeeping gene HPRT1. The sequence of the reverse primer CD19 was 5′AAGTGTCACTGGCAGTATACAC and the sequence of the reverse primer HPRT1 primer was 5′GGTCTCTTTTCGCAAGCAA. For reverse-transcription reaction samples were incubated at 60 minutes at 42 degrees instead of 15 minutes at 37 degrees.

Reverse Transcription, Polymerase Chain Reaction (PCR), and Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

cDNA samples were amplified by semiquantitative PCR using Taq-DNA polymerase I (Axon Labortechnik, Kaiserslautern, Germany) and the CD19 isoforms were visualized in 1.2% agarose gels. Primers used for amplification of the CD19 isoforms are described in Sitollo et al.17 Quantitative real time polymerase chain reaction (qRT-PCR) was performed using KAPA SYBR FAST MasterMix (PeqLab, Erlangen, Germany) and exon/exon junction-specific oligo pairs. The specific primer pair for the Δex2-isoform has already been described.17 The sequence of the forward primer of the ex2-part-isoform was 5′GCCCTCTCTTCTCTCTCTTTT and the sequence of the reverse primer was 5′CCCGAACAAGCTCCCCCTTCACCTTC. The forward primer used for HPRT1 was 5′TGAACCTGGAAAAAATATGCA and the reverse primer was 5′GGTCTCTTTTCGCAAGCAA. The expression of CD19 was analyzed with primers spanning exons 8 and 9. The forward primer was 5′TGCCCCGTCTTATGGAAAC and the reverse primer was 5′CTCTTCTCTGGGCCGACTCA. After normalization to the housekeeping gene HPRT1, the relative quantification value was expressed as 2−ΔΔCt. The calibrator was calculated as the maximal number of cycles used in the PCR (40) minus the mean of the HPRT1 Ct values, resulting in a value of 19.

TA Cloning and Sequencing

TA cloning was performed by TA Cloning Kit, with PCR2.1 Vector and One Shot TOP10F+ Chemically
Competent *Escherichia coli* (Thermo Scientific) following the manufacturer’s instructions. Lysogeny broth plates used contained X-Gal, isopropyl β-D-1-thiogalactopyranoside, and ampicillin. Clones were analyzed by Sanger sequencing. PCR products were purified by an enzymatic method using 10IU exonuclease I and 2IU shrimp alkaline phosphatase (New England Biolabs, Frankfurt, Germany) for 30 minutes at 37°C and 15 minutes at 80°C and sequenced by using ABI Prism 3100 Genetic Analyzer and the BigDye v3 Terminator Kit (Thermo Scientific). The sequences were compared with the reference sequence using the Sequencher program (Gene Codes).

## RESULTS

### Sample Characteristics and Cohort

In the pediatric B-ALL group, analysis involved 11 bone marrow samples and 4 peripheral blood samples. Both, bone marrow and peripheral blood were only available from 1 child. Of all bone marrow samples, 9 were retrieved when the children initially came to the hospital. One sample was retrieved on day 29 of the leukemia therapy protocol meaning the child had already been treated with conventional chemotherapy. Another sample was taken when a first relapse of the child was initially diagnosed. All of the peripheral blood samples were retrieved when the children received their initial leukemia diagnosis. Samples of the control group were all bone marrow samples. Median age of the leukemic patients was 4 years. Median age of the control group was 5.5 years. In total, 30.8% of the leukemic patients were female, whereas the control group was only male. Statistically, boys are minimally more affected by the disease than girls. In total, 46.2% of the leukemic patients were diagnosed with pre-B-ALL, whereas the other 53.8% were diagnosed with common B-ALL.

In the adult B-ALL group, analysis involved 6 bone marrow samples that were retrieved when the patients received their initial diagnosis. Median age of the adult patients was 54 years. In total, 66.7% of the adults were male. A total of 50% of the patients were diagnosed with common B-ALL, whereas 33.3% were diagnosed with pro-B-ALL and only 1 patient (16.7%) was diagnosed with pre-B-ALL. All patients’ and controls’ characteristics are summed up in Tables 1–3. The percentage of blasts as detected during the standard diagnostic and the quality of the extracted RNA are also reported as RNA Integrity Number (RIN) value.

### CD19 Isoforms Lacking the CART-19 Epitope Exist Before Any Therapy in Children and Adults

To study the presence of CD19 mRNA isoforms, we analyzed bone marrow as well as peripheral blood samples by semi-quantitative RT-PCR amplification of CD19 cDNA and visualized them by agarose gel electrophoresis. Amplifying exons 1–5 in 4 of our pediatric leukemia sam-

| Patients | Sex | Age (y) | Diagnosis | Site | Time of Sampling | RIN | Leukemic Blasts in Sample (%) | Leukemic Blasts | Percentage CD19% Blasts | Relative Expression ex2part (%) | Relative Expression Δex2 (%) |
|----------|-----|---------|-----------|------|-----------------|-----|-----------------------------|----------------|--------------------------|-------------------------------|-----------------------------|
| P1       | F   | 9       | Pre-B-ALL | BM   | Initial blasts  | 7.5 | 76                          | 98             | 45.69                    | 2.76                          |                             |
| P2       | M   | 0       | Pre-B-ALL | BM   | Initial blasts  | 6.3 | 57                          | 99             | 32.53                    | 12.41                         |                             |
| P3bm     | M   | 4       | Common B-ALL | BM | Initial blasts  | 7.4 | 73                          | 99             | 24.83                    | 6.34                          |                             |
| P3pb     | M   | 3       | Pre-B-ALL | PB   | Initial blasts  | 7.4 | 68                          | 99             | 30.35                    | 13.58                         |                             |
| P4       | M   | 3       | Common B-ALL | BM | Initial blasts  | 6.4 | 80                          | 99             | 16.96                    | 5.59                          |                             |
| P5       | M   | 7       | Common B-ALL | PB | Initial blasts  | 7.1 | 14                          | 98             | 32.99                    | 12.50                         |                             |
| P6       | M   | 7       | Common B-ALL | BM | Day 29 of therapy | 7.3 | 4                           | 0.2             | 10.51                    | 4.94                          |                             |
| P7       | M   | 9       | Common B-ALL | PB | Initial blasts  | 7.7 | 40                          | 86             | 16.96                    | 5.59                          |                             |
| P8       | M   | 1       | Pre-B-ALL | PB   | Initial blasts  | 7.2 | 59                          | 77             | 16.27                    | 26.61                         |                             |
| P9       | M   | 4       | Common B-ALL | BM | Initial blasts  | 6.4 | 42                          | 99             | 1.91                     | 1.39                          |                             |
| P10      | M   | 17      | Common B-ALL | BM | Relapse         | 5.9 | 76                          | 99             | 7.64                     | 3.52                          |                             |
| P11      | M   | 17      | Common B-ALL | BM | Initial blasts  | 5.8 | 66                          | 98             | 7.91                     | 3.30                          |                             |
| P12      | F   | 15      | Pre-B-ALL | BM   | Initial blasts  | 5.7 | 60                          | 96             | 1.61                     | 0.67                          |                             |
| P13      | F   | 4       | Pre-B-ALL | BM   | Initial blasts  | 9   | 77                          | 98             | 30.57                    | 11.99                         |                             |
| P14      | F   | 4       | Common B-ALL | BM | Initial blasts  | 6.0 | 62                          | 17             | 9.94                     | 4.39                          |                             |

The quality of the extracted RNA is reported as RIN value. The relative expression of the isoforms ex2part and Δex2 is calculated as in Figure 1C. B-ALL indicates B-cell acute lymphoblastic leukemia; BM, bone marrow; F, female; M, male; PB, peripheral blood; RIN, RNA Integrity Number.
The apparent higher expression of the isoforms in all pediatric samples except for 1 leukemia patient and 1 D isoform is expressed more strongly than the (Fig. 1C). Furthermore, our results show that the ex2part-expressed pediatric leukemia patients as well as in our control group (Fig. 1D). These results indicate that the expression of exons 8 and 9 representing the specific splicing variant exon/exon junctions and compared we performed qRT-PCR analysis using oligos spanning the detected are indeed the ex2part-isoform and whole this to the expression of exons 8 and 9. The ex2part-isoform implies a partial deletion of exon 2, whereas the D isoform involves a complete deletion of exon 2.1 The wildtype was visible in all samples and had the strongest expression. The ex2part-isoform and D isoform were slightly expressed in all samples. Similar results were obtained in samples of nonleukemia patients (Fig. 1B). The results of amplifying exons 1–5 in all 6 adult leukemia samples were identical to the results of the pediatric samples (Fig. 1D). These results indicate that isoforms that contribute to resistance to CART-19 therapy.

To analyze the expression of both isoforms in a larger number of leukemia patients and confirm that the bands detected are indeed the ex2part-isoform and D isoform, we performed qRT-PCR analysis using oligos spanning the specific splicing variant exon/exon junctions and compared this to the expression of exons 8 and 9 representing the whole CD19 gene (for the elucidation of the sequence of CD19 ex2part see below). Both, the ex2part-isoform and D isoform were expressed at different levels in all 6 adult leukemia samples identical to the results of the pediatric samples (Fig. 1D). These results indicate that isoforms that contribute to resistance to CART-19 therapy by losing the CD19 epitope FMC63 preexist at initial leukemia diagnosis before any kind of therapy.

The quality of the extracted RNA is reported as RIN value. The relative expression of the isoforms ex2part and D is calculated as in Figure 1C. BM indicates bone marrow; M, male; RIN, RNA Integrity Number.

| Patients | Sex | Age at Sampling Point | Diagnosis | Sample | RIN | Relative Expression ex2part (%) | Relative Expression D (%) |
|----------|-----|-----------------------|-----------|--------|-----|----------------------------------|--------------------------|
| C1       | M   | 1                     | BM        | 5.2    | 30.15 | 12.50                            |
| C2       | M   | 16                    | BM        | 4.3    | 72.70 | 25.35                            |
| C3       | M   | 8                     | BM        | 6.1    | 21.17 | 30.15                            |
| C4       | M   | 2                     | BM        | 5.8    | 49.31 | 32.53                            |
| C5       | M   | 2                     | BM        | 7.1    | 50.35 | 19.34                            |
| C6       | M   | 6                     | BM        | 4.2    | 51.76 | 23.82                            |

The quality of the extracted RNA is reported as RIN value. The relative expression of the isoforms ex2part and D is calculated as in Figure 1E. BM indicates bone marrow; M, male; RIN, RNA Integrity Number.

Skiping of exons 5 and 6 affects the expression of the transmembrane and cytosolic domains of the CD19 protein. Amplifying exons 4–8 in our pediatric samples resulted in 2 different bands (Fig. 2A). The strongest band matched the size of the full-length isoform which could be seen in all our samples. The greater band of about 800 bp has not been described so far and was clearly visible in 10 of the 15 samples. Amplification by the exon 4–8-specific primer in healthy children resulted in the same 2 different bands as described previously (Fig. 2B). In adult patients, the full-length isoform was visible in all patients and had the strongest expression, whereas the so far unknown band of about 800 bp was expressed in 5 of the 6 patients (Fig. 2C). It
is interesting to note that we detected an isoform of 331 bp in the single adult patient that did not express the 800 bp band (sample A5). This patient was diagnosed with common B-ALL. This isoform was consistent in size with the $\Delta$ex5–6 isoform described by Sotillo and colleagues in patients who had been treated with either blinatumomab or CART-19 therapy and became resistant to those therapies. Furthermore, the adult patients showed another 2 bands that were slightly expressed and have not been described so far. One band matched the size of about 500 bp, whereas the other one involved about 550 bp (Fig. 2C). Our results indicate that the expression of isoforms especially concerning the region between exons 4 and 8 in adult leukemic patients may differ from pediatric patients at the time of diagnosis. Therefore, it will be interesting to clarify the sequences of the unknown bands to better understand why the expression of isoforms differs between adults and children and what this means in terms of CAR therapy resistance.

**CD19 Isoforms Lacking the CART-19 Epitope Are Specifically Expressed in Leukemic Blasts**

As bone marrow and peripheral blood samples also contain other hematopoietic cells besides leukemic blasts, we validated the presence of CD19 isoforms lacking the CART-19 epitope in leukemic blasts by cell sorting. Antibodies used were anti-CD19, anti-CD45, and 7-AAD to exclude dead cells. CD19 is specifically expressed on B cells as well as on follicular dendritic cells. Both, B cells and dendritic cells are CD45$^+$ but located in different gates due to their granularity when gating the side-scatter against CD45. The different populations of B cells and leukemic blasts can be detected by CD45 positivity. Mature B cells carry CD45 on
their surface, whereas leukemic blasts are usually CD45^- or weakly positive. Nearly 100% of the blast population was CD19+. Cell sorting was performed for 3 of our pediatric as well as 2 of our adult samples. The gating strategy for the cell sorting of the leukemic blasts and corresponding FACS data are listed in Supplemental Fig. 1, Supplemental Digital Content 1, http://links.lww.com/JIT/A473.

Expression analysis by qRT-PCR using the same oligos as described previously revealed similar results compared with the unsorted samples. The ex2part-isoform and Δex2-isoform were still expressed at different levels in both the pediatric and the adult patients and the ex2part-isoform was still expressed more strongly in all samples than the Δex2-isoform (Fig. 3A). The apparent higher expression in the unsorted samples compared with the corresponding sorted samples derives from the lower expression of CD19 ex8–9 in the sorted samples. This can be explained by the fact that unsorted samples still contain CD19+ B cells. By showing that the results of the sorted and unsorted samples are identical in terms of the general expression of CD19 isoforms, we proved that the CD19 isoforms described previously are specific to leukemic blasts.

Amplification of exons 4–8 in the sorted samples also resulted in the same bands as in the corresponding unsorted samples described previously (Figs. 3B, C).

The CD19 Ex2part-Isoform Leads to a Truncated Protein

Sotillo et al.\(^{17}\) described the ex2part-isoform in their work but the sequence was not disclosed. To further characterize this isoform, we performed TA cloning and found a deletion comprising 131 bp (Fig. 4A), which leads to a frameshift within exon 2 (Fig. 4B). At first sight, the deletion seems to be already known as the SIB Genes Track implemented in the UCSC Genome Browser mentions an isoform with a partially deleted exon 2. However, comparison of the 2 sequences reveals an extra guanine in our sequence. Compared with the RefSeq sequence of exon 2, the frameshift leads to a premature integration of a stop codon which results in a truncated protein ending in exon 2. Another possibility is a delayed start of the protein in exon 3 and therefore loss of the FMC63 epitope that is recognized by CAR T cells. According to the UCSC Genome Browser, acetylation of H3K27 (H3K27ac) and DNase 1 hypersensitive peaks which marks both active enhancers and active promoters\(^{29}\) can also be found between exon 1 and 3 (Fig. 4C) suggesting that the protein may start from other exons as well.

**DISCUSSION**

Our study addresses the important clinical issue of resistance to CART-19 therapy and establishes the preexistence of CD19 isoforms as a possible general mechanism of escape. Our results suggest that some of the CD19 isoforms previously detected in patients who became resistant to CART-19 therapy\(^{17}\) are not leukemia specific, already preexist at diagnosis and might evolve during therapy as a dominant clone. Our results partially explain the relative high rate of relapse under CART-19 therapy suggesting...
that combination therapies addressing several epitopes could be necessary to achieve a long-term complete remission in these patients. Moreover, our results indicate that more CD19 isoforms than previously known exist. These isoforms might be even specific to adults or children.

Alternative splicing increases protein diversity and plays an important role not only in human development and tissue identity but also in diseases such as cancer. Cancer cells are able to use alternative splicing variants to escape targeted therapy. This escape mechanism has been described in several cancer types. For instance, in breast cancer, a splicing variant that eliminates exons 16 in the extracellular domain of HER2 contributes to resistance to trastuzumab.23 Furthermore, melanoma cells may become resistant to vemurafenib by dimerization of aberrantly spliced BRAF (V600E).24

In oncohematology, resistance to rituximab therapy has been associated with the presence of an alternative CD20 transcript lacking the major part of the transmembrane and extracellular domains including the rituximab epitope.23 This transcript has been observed in malignant cells but not in peripheral blood mononuclear cells, bone marrow-derived mast cells or plasmocytes from healthy donors.23

In contrast to the CD20 isoform described previously, our results show that CD19 isoforms lacking exon 2 are already expressed in leukemic blasts at the time of diagnosis. More importantly, these isoforms are also expressed in the bone marrow of nonleukemia donors. Alternative CD19 splicing may have consequences on the CD19 protein function that may influence cell signaling and finally B-cell function at different stages of B-cell maturation. According to recent studies based on a transcriptome analysis performed throughout normal hematopoiesis, specific splicing events occur at defined stages of hematopoiesis leading to a selective inclusion of individual exons which alter the function and/or stability of the encoded proteins to define a cell’s identity (for review see Inoue et al24). As we found the same CD19 splicing variants in both, the leukemia and the control group, these variants are possibly required for the regulation of CD19 function and are not pathogenic variants. Importantly, it has been shown that the CD19 isoform lacking exon 2 can partially replace the wild-type CD19,17 implicating that a selection of this isoform under therapy pressure is virtually possible in each patient. The isoform lacking a part of exon 2 results in a premature stop codon. As a consequence, the mRNA may be deleted by nonsense-mediated mRNA decay, a pathway dedicated to the destruction of transcripts containing a premature termination codon, which also plays an important role in controlling the expression of naturally occurring transcripts.25 However, another possibility is a delayed start of the protein in exon 3 with the loss of the extracellular domain encoded by exon 1 and 2 and therefore the epitope that is recognized by CART-19 therapy. The function of the shorter protein remains to be elucidated.

In contrast to the isoform lacking exon 2, which is equally expressed in children and adults at the time of diagnosis, the Δex5–6 isoform lacking the transmembrane and cytosolic domains was not expressed in either our pediatric leukemic blasts at the time of diagnosis or in the bone marrow of our nonleukemia donors. As skipping of exons 5 and 6 is considered to lead to a lack of the transmembrane and cytosolic domains of the CD19 protein and therefore contributing to immune evasion of the tumor cells under therapy, the Δex5–6 isoform in children possibly develops only under CAR-therapy pressure. However, we identified the Δex5–6 isoform in 1 adult patient with common B-ALL at the time of diagnosis suggesting that this isoform can exist before any therapy. Owing to the small number of patients analyzed in this study, we cannot conclude if there is a difference in the expression pattern of the Δex5–6 isoform between children and adults at the time of diagnosis.

Our data show that both isoforms lacking the CART-19 epitope exist before any kind of therapy which indicates that leukemic blasts already command the basis for developing CART-19 therapy resistance from the outbreak of the disease. Even if the leukemic blasts just weakly express these shorter CD19 isoforms, it is likely that their expression increases during CART-19 therapy as shown by Sotillo et al17 leading to therapy resistance as well as relapses of the leukemia. At the moment, we cannot discriminate if blast clones exist that only carry a particular isoform or if the isoforms are expressed together. The consequences of the selection would be the same for both scenarios, that is the selection of blast cells which rely on a therapy-invisible CD19 isoform for their growth.

Our results suggest that biomarkers that predict the preferential selection of preexisting CD19 isoforms may become relevant in the early identification of CART-19 refractory patients. For this, it will be important to dissect the molecular determinants of CD19 exon splicing and correlate them to the therapeutic outcome. The determinants of CD19 isoforms enabling resistance to CART-19 are shown in FIGURE 3. CD19 isoforms lacking the CART-19 epitope are specifically expressed in leukemic blasts. A. Expression of the CD19 ex2part-isoform, the Δex2-isoform, and CD19 ex8–9 in 3 pediatric (P) and 2 adult patients (A) after cell sorting was analyzed by qRT-PCR. The percentage of the expression of the isoforms relative to CD19 ex8–9 is shown. Expression analysis was done in triplicates. Expression of CD19 isoforms was analyzed by polymerase chain reaction with a primer pair amplifying exon4–8 in 3 pediatric leukemia patients (B) and 2 adult leukemia patients (C). The full-length (fl) plus a new isoform of 800 bp are indicated. The position of the expected Δex5–6 isoform is also indicated. The size of 500 bp is indicated for adult patients. The numbers of the pediatric patients (P) and adult patients (A) according to Tables 1 and 3 are shown. bm indicates bone marrow.
exon splicing can include mutations and single nucleotide polymorphisms that affect exon skipping and change the expression level in splicing factors. In oncohematology, numerous mutations in genes encoding proteins involved in mRNA splicing (particularly SF3B1, U2AF1, and SRSF2) have been identified in chronic lymphocytic leukemia, myelodysplastic syndromes, and lymphomas but are rare in ALL. The SRSF3 splicing factor regulates the inclusion of CD19 exon 2 and SRSF3 insufficiency in relapsed leukemia could be at least partly responsible for the abundance of the CD19 Δex2-isoform. Moreover, in contrast to the isoform lacking exon 2, the Δex5–6 isoform is not expressed in all patients at the time of diagnosis and its preexistence might be a potential predictor of CART-19 therapy response. Finally, the expression level of the isoforms at the time of diagnosis may also correlate with the development of resistance.

In conclusion, our results underline the risk of using CART-19 therapy as monotherapy and suggest the necessity to identify predictive biomarkers and new (tumor specific) epitopes as targets for combination therapies. Combination therapy with chimeric receptors against CD123 has been recently shown to prevent antigen-loss relapses occurring after CD19-directed therapies.

CONFLICTS OF INTEREST/FINANCIAL DISCLOSURES

This publication contains data from the medical dissertation of J.F.

All authors have declared that there are no financial conflicts of interest with regard to this work.

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