Acute myeloid leukemia early after anti-CD19 chimeric antigen receptor T cells infusion in a patient with refractory diffuse large B cell lymphoma and pre-existing clonal hematopoiesis

by Lorenza Falini, Alessandra Venanzi, Valentina Tini, Alessandra Innocente, Stelvio Ballanti, Simonetta Saldi, Silvio Sivolella, Antonio Pierini, Cynthia Aristei, Enrico Tiacci, Vincenzo Maria Perriello, and Brunangelo Falini

Received: May 3, 2022.
Accepted: July 15, 2022.


Publisher's Disclaimer. E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication. E-publishing of this PDF file has been approved by the authors. After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors' final approval; the final version of the manuscript will then appear in a regular issue of the journal. All legal disclaimers that apply to the journal also pertain to this production process.
Acute myeloid leukemia early after anti-CD19 chimeric antigen receptor T cells infusion in a patient with refractory diffuse large B cell lymphoma and pre-existing clonal hematopoiesis

Lorenza Falini1*, Alessandra Venanzi1*, Valentina Tini1, Alessandra Innocente1, Stelvio Ballanti1, Simonetta Saldi2, Silvio Sivolella3, Antonio Pierini1, Cynthia Aristei2, Enrico Tiacci1, Vincenzo Maria Perriello1^, Brunangelo Falini1^  

1 Institute of Hematology, Department of Medicine and Surgery, Center for Hemato-Oncological Research (CREO), University of Perugia, Perugia, Italy  
2 Radiation Oncology Section, Department of Medicine and Surgery, University of Perugia, Perugia, Italy  
3 Nuclear Medicine, Foligno Hospital, Foligno, Italy

*Equal contribution  
^Co-last authors

Correspondence to: brunangelo.falini@unipg.it

Contributions: LF, VMP, ET and BF conceived and designed the study; LF, AI, SB, SS, AP and VMP managed the patient; VMP and BF carried out the pathological analysis; AV and VT carried out the NGS analysis; SS and CA carried out the radiotherapy; VMP and BF wrote the manuscript; CA and BF approved the final draft of the manuscript.

Acknowledgments: we thank Prof. Stefano Lazzi for performing FISH analysis on lymph node biopsy.

Data sharing statement: the data that support the findings of this study are available from the corresponding author, upon reasonable request.
Immunotherapy with CD19-directed chimeric antigen receptor (CAR) T cells (tisagenlecleucel, axicabtagene and lisocabtagene) has revolutionized the treatment of de novo and transformed relapsed/refractory (R/R) DLBCL, inducing long-term complete response (CR) in about 40% of cases\textsuperscript{1-3}.

The majority of patients candidate to CD19 directed CAR-T cells for R/R DLBCL have been previously exposed to many anti-tumor agents, including chemotherapy drugs, conjugated monoclonal antibodies and radiation, administered to achieve CR during the course of the disease or as a bridging therapy to CAR-T cells infusion. Chemoradiotherapy is well known to exert a genotoxic effect favoring the occurrence of secondary tumors, including myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML)\textsuperscript{4}. Myeloid malignancies have been so far occasionally reported after CD19-directed CAR-T cell therapy of B-cell acute lymphoblastic leukemia (B-ALL). They include the lineage switch of MLL-rearranged B-ALL into a clonally related AML under the immune pressure of anti-CD19 CAR-T cells\textsuperscript{5} or the development of a clonally unrelated new myeloid neoplasm\textsuperscript{6}. On the other hand, no information on development of myeloid neoplasms in DLBCL patients treated with CD19-directed CAR-T cells is to our knowledge available.

Here, we present the first case of AML developing soon after CAR-T cells infusion in a R/R DLBCL patient.

A 69-year-old women was diagnosed with a follicular lymphoma, grade 2 in 2016. A positron emission tomography/computerized tomography (PET/CT) showed involvement of almost all superficial, thoracic and abdominal lymph nodes (LNs), the presence of two subcutaneous nodules and a pleural effusion. The bone marrow (BM) biopsy was not involved by lymphoma. The blood cell count (BCC) was: WBC 17.690/mm\textsuperscript{3} (neutrophils 83%), Hb 14 gr/dl, platelets 459.000/mm\textsuperscript{3}. She received 6 cycles of bendamustine plus rituximab, achieving a near CR (persistence of a few neck LNs). Three months later, she relapsed in all nodal and extranodal sites initially involved by the disease. A new LN biopsy revealed a DLBCL transformed from follicular lymphoma, without MYC, BCL2 and BCL6 rearrangements. Therefore, the patient underwent 6 cycles of R-COMP plus prophylaxis for central nervous system-CNS involvement, achieving a CR that only lasted three months. Then, she received one cycle of R-DHAOX (rituximab, dexamethasone, cytarabine and oxaliplatin) followed by collection of hematopoietic stem cells (HSCs) and autologous hematopoietic stem cell transplantation (HSCT) using FEAM (fotemustine,
etoposide, cytarabine and melphalan) as conditioning regimen. This resulted into a CR but, 5 months later, the PET/CT scan showed reappearance of the disease in all nodal and extranodal sites. The patient started with lenalidomide (10 mg/die x 3 weeks) plus prednisone, achieving CR after 5 cycles. The BCC in November 2018 was: WBC 7.000/mm$^3$ (neuthrophils 78%), Hb 11.7 gr/dl, platelets 115.000/mm$^3$. She proceeded with additional 4 cycles of lenalidomide plus prednisone that were stopped because of severe pancytopenia. The BM biopsy, performed one month after Lenalidomide discontinuation, showed an hypocellular marrow consistent with previous myelotoxic therapy but no neoplastic infiltration. However, a disease progression in all nodal and extranodal sites was documented at PET/TC scan. A submandibular LN biopsy (June 2019) showed involvement by DLBCL expressing all B-cell markers, including CD19. The patient started again lenalidomide plus steroids achieving a CR that lasted about 1.5 years. In March 2021, PET/CT showed again disease progression (Figure 1A). Thus, the patient was regarded as eligible for CD19-directed CAR-T cells and leukapheresis was performed. The blood cell count was: WBC 2380/mm$^3$ (neuthrophils 60%), Hb 11.2 gr/dl, platelets 69.000/mm$^3$. The BM biopsy showed an hypocellular marrow that was interpreted as due to previous therapies. As bridging therapy to CAR-T cells, she received the HAM regimen (mitoxantrone and cytarabine) followed by reinfusion of autologous HSCs achieving partial remission.In the following months, several episodes of cytomegalovirus (CMV) reactivation prevented to proceed with CAR-T cell infusion and therefore in July 2021 we decided to control the disease through a total lymphoid radiation (TLI) for 5 days. In particular, all the main nodal station received 15 Gy in 10 fractions. A simultaneous integrated boost (2 Gy up to 20Gy) was delivered to the active disease.

In August 2021, the BCC was: WBC 4310/mm$^3$ (neuthrophils 56%), Hb 10.6 gr/dl, platelets 80.000/mm$^3$ and the BM biopsy showed an hypocellular marrow without clear MDS or neoplastic infiltrates (Figure 1C) with a normal percentage of CD34+ cells (Figure 1D). Therefore, she received infusion of CD19-directed CAR-T cells after lymphocyte depletion with fludarabine and cyclophosphamide. No cytokine release syndrome (CRS) or neurotoxicity was recorded. CD19 CAR-T cell expansion was detectable by flow-cytometry from day 7 to 21. PET/CT scan performed at 1 and 3 months after CAR-T cells showed a CR (Figure 1B). Because of persistent severe pancytopenia from lymphodepleting therapy (WBC 450/mm$^3$, Hb 8 g/dL, platelets 6.000/mm$^3$ on 27/10/21) lasting 60 days after CAR-T cells infusion and requiring transfusion support, a new BM biopsy and aspirate was performed that showed an hypocellular marrow infiltrated by 40-50% blast cells with
myelomonocytic appearance expressing CD34 (Figure 1E), myeloperoxidase and CD68, and frequently exhibiting erythrophagocytosis (Figure 1 F). No normal or neoplastic B cells were detected in the BM by flow cytometry and immunohistochemistry. Cytogenetic showed a complex karyotype: 45,xx,-7, del(11)(p15) [8]/46,idem, t(2;19) (p12;q13.3)[6]/46,XX[3]. Targeted sequencing detected mutations of the following genes: DNMT3A V626GfsTer4 (VAF:46.2%), RUNX1 splicing-site mutation (VAF: 16.8%) and missense mutation N136K (VAF 9.2%), and PPM1D S453* (VAF1.4%). Targeted sequencing of stored DNA from the BM samples taken before CAR-T cells was also performed, already showing the PPM1D mutation but not RUNX1 mutations (Figure 2). We could also retrospectively analyze the kariotype from a BM aspirate taken in March 2021, already showing the following karyotype: 45,XX,-7, del(11)(p15)[8]/46,XX[12]. Given the diagnosis of AML, while in CR for DLBCL, we decided to start the patient on 5-Azacitidine plus Venetoclax as bridge to a potential allogenic stem cell transplantation. However, Venetoclax was stopped after the first cycle due to persistent pancytopenia and a BM evaluation performed after 2 cycles of 5- Azacitidine showed AML persistence. After 4 cycles of 5-Azacitidine pancytopenia is continuing while the patient is still in complete remission for DLBCL at 6 months from CAR-T cell therapy.

Our patient showed persistent severe pancytopenia after CAR-T cell therapy. Pancytopenia of various degree frequently occurs in the first 1-3 months after infusion of CD19-directed CAR-T cells and may be ascribed to several conditions, including bridging and/or lymphodepletive therapy administered before CAR T-cells, cytokines release by CAR-T cells, BM involvement by lymphoma or leukemia, BM failure related to drugs other than CAR-T cells and/or infectious complications (e.g. CMV)7-8. Among these causes, BM pancytopenia due to BM involvement by lymphoma was unlikely in our patient because it was never documented during the previous 5 years of disease. Similarly, no infectious complications could be recorded after CAR-T cells. Instead, myelotoxicity due to bridging chemoradiotherapy (HAM regimen and TLI) could have played a role in the persistent pancytopenia of our patient. In order to discriminate between these possibilities, we performed a BM biopsy 60 days after CAR-T cell therapy, that surprisingly revealed AML with complex karyotype, including monosomy 7. This diagnosis was in a way unexpected since a BM biopsy taken just before CAR-T cells infusion showed no evidence of MDS/AML.
The pathogenesis of AML in our case remains unclear. Clonal expansion of CAR-T cells due to unintentional lentiviral vector mediated insertion of the CAR transgene in the \textit{TET2} or \textit{ABL} gene has been reported\textsuperscript{9,10}. However, the possibility that AML could have developed following unintentional insertion of CAR transgene into AML-associated genes in a contaminating HSC during manufacturing was excluded by flow-cytometry, showing CAR expression only on T lymphocytes inside all peripheral blood population, thereby excluding the presence of any possible circulating CAR positive myeloid cells. According to the World Health Organization (WHO) classification of myeloid neoplasms, the leukemia in our patient was consistent with a therapy-related AML (t-AML), because of previous history of chemo-radiotherapy and the complex karyotype. t-MDS/AML has been ascribed for a long time to DNA mutations and chromosome breakage induced in HSCs by ionizing radiation and/or genotoxic drugs (e.g. alkylating agents or topoisomerase 2 inhibitors). Instead, it is now clearly emerging that these stressors of hematopoiesis can promote expansion of pre-existing mutant clones driven by clonal hematopoiesis (CH)\textsuperscript{11}.

CH has been reported in 48\% of patients receiving CAR-T cell therapy for B-cell non-Hodgkin lymphomas or multiple myeloma but, unlike CH found in patients pre-autologous HSCT\textsuperscript{12}, it was not associated with a worse survival, including increased risk of death from t-MDS/AML\textsuperscript{13}. Mutations of DNA damage response genes \textit{TP53} and \textit{PPM1D}, typically observed in therapy-related CH\textsuperscript{14}, were not detected in our patient since \textit{TP53} was germline while the \textit{PPM1D} mutation, despite developing (at a VAF of 1.1\%) before leukemia onset, remained at a similar low frequency (VAF 1.4\%) at leukemia onset without seeding the tumor clone (which had RUNX1 mutations at higher VAFs, up to 16.8\%). In contrast, CH in our patient was mainly driven by \textit{DNMT3A} that was already present in 2016, at the time of lymphoma diagnosis. DNMT3A driven CH has been shown to be by itself predictor for AML development\textsuperscript{11,14}. In contrast, \textit{RUNX1} mutations were detected for the first time in the BM sample taken 2 months after CAR-T cells, pointing to \textit{RUNX1} mutations as drivers in promoting t-AML in our patient\textsuperscript{15}, probably through cooperation with deletion of monosomy 7. Because there was no evidence of AML in the BM taken just before CAR-T cells infusion, we hypothesize that AML may have developed as consequence of the immunosuppression related to lymphodepletion pre-CAR-T cell infusion, although we cannot exclude that it could be related to effect previous multiple genotoxic treatments (e.g. anthracyclines, radiotherapy or autologous stem cell transplantation). However, studies of similar cases are required to better clarify this issue. For the time being, we recommend CD34 immunostaining as well as NGS and cytogenetic
analysis in the BM of heavily pre-treated DLBCL patients before CAR-T therapy, especially if they have evidence of cytopenia than can be erroneously interpreted as BM marrow hypoplasia related to previous therapy. Considering higher risks to develop t-AML, such patients should be carefully evaluated in order to decide if they could be excluded from CAR-T cell therapy favoring immunotherapy approaches not requiring lymphodepletion, as bispecific or drug-conjugated antibodies.
References
15. Christiansen DH, Andersen MK, Pedersen-Bjergaard J. Mutations of AML1 are common in therapy-related myelodysplasia following therapy with alkylating agents and are significantly associated with deletion or loss of chromosome arm 7q and with subsequent leukemic transformation. Blood. 2004;104(5):1474-1481.
**Figure legends**

**Figure 1. PET-CT and bone marrow examination before and after CAR-T cells infusion.** A,B) FDG-PET/CT coronal maximum intensity projection (MIP) images before leukapheresis with avid uptake of abdominal lymph nodes (A) and 3 months after CD19-directed CAR-T cell therapy (B), showing metabolic complete response of DLBCL. C) BM biopsy taken before CAR-T cells infusion showing a hypocellular marrow without leukemic infiltration (Hematoxylin and eosin; x 400). D) The same sample as Figure 1 C showing only rare CD34+ cells within the normal range (red arrow); vessel endothelial cells serve as positive control (black arrow) (immunoperoxidase, diaminobenzidine; x 200). E) BM biopsy taken 2 months after CAR-T cells infusion showing an hypocellular marrow infiltrated by CD34+ leukemic cells (red arrows); vessel endothelial cells serve as positive control (black arrows) (immunoperoxidase, diaminobenzidine; x 200). F) BM smear 2 months after CAR-T cells infusion showing myeloid blasts exhibiting erythrophagocytosis (black arrows) (May-Grunwald-Giemsa, x 400).

**Figure 2. Clonal evolution from Clonal Hematopoiesis (CH) to therapy related AML**

The fish plot shows the inferred clonal evolution pattern based on targeted sequencing. The phylogenetic trees visualize the estimated order of mutation acquisition and the proportion of subclones with a different combination of mutations at each timepoint. The PPM1D mutation has been depicted as presumptively subclonal within the DNMT3A-mutant pre-leukemic clone, but its low VAF would be compatible also with the existence of a separate PPM1D-mutant CHIP clone independent from (i.e., outside of) the DNMT3A-mutant pre-leukemic clone. RUNX1 missense mutation has been presumptively depicted as subclonal within the RUNX1 splice-site mutant leukemic clone. R-BENDA: rituximab, bendamustine; R-CMP: Rituximab, Cyclofosfamide, non-pegylated liposomal doxorubicin, prednisone; R-DHAOX: rituximab, high dose ARAc, Oxaliplatin; ASCT, preceded by fotemustine, etoposide, ARAc and Melphalan; R-Lenalidomide, Rituximab-Lenalidomide; HAM, High dose ARA-C and Mitoxantrone; TLI, Total Lymphonodal Irradiation.