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Authors and affiliations

David A. Sallman1*, Jason Brayer1*, Elizabeth M. Sagatys2, Caroline Lonez3, Eytan Breman3, Sophie Agaugué3, Bikash Verma4, David E. Gilham3, Frédéric F. Lehmann3, Marco L Davila5

1Malignant Hematology and 2Hematopathology and Laboratory Medicine, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA

3 Celyad, SA, Mont-Saint-Guibert, Belgium

4 Celyad, SA, New York, NY, USA

5Blood & Marrow Transplantation and Cellular Immunotherapy, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA

*These authors contributed equally to this work.

Contact information for correspondence: David A. Sallman or Marco L Davila, H. Lee Moffitt Cancer Center, Tampa, FL, USA. Phone: 1-888-663-3488, Email: David.Sallman@moffitt.org or Marco.Davila@moffitt.org

Running heads: Remission in an r/r AML patient with a CAR-T

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Acute Myeloid Leukemia (AML) is the most common acute leukemia affecting adults characterized by the accumulation of immature myeloblasts in the marrow or peripheral blood. Natural Killer Group 2D (NKG2D) is an activating receptor expressed on Natural Killer (NK) cells and activated CD8\(^+\) T cells, which triggers cytotoxicity following recognition of its ligands (namely MHC class I polypeptide-related sequence A and B (MICA/B) and unique long protein 16 (UL-16) binding protein 1 to 6 (ULBP1 to 6)) at the surface of stressed, infected and most notably transformed cells \(^1\). Consequently, a broad range of primary tumors express NKG2D ligands (NKG2DL) and can be targeted by NKG2D-based immunotherapies \(^2\) \(^3\). NKG2DL expression was reported in AML, with data reporting 67% to 100% expression in MICA/B and/or ULBP2-3 expression in blasts \(^4\) \(^5\) with all blasts expressing at least one ligand [Dulphy N, Toubert A et al., unpublished data].

CYAD-01 are autologous T-cells genetically modified to express a chimeric antigen receptor (CAR) comprising a fusion of the human full-length NKG2D receptor with the CD3\(\zeta\) signaling domain \(^6\). Although the co-stimulatory molecule DNAX-activating protein 10 (DAP10) is not part of the transgene, NKG2D associates with this molecule for membrane stabilization to provide the secondary activation signal \(^1\). The NKG2D-CAR construct binds 8 different ligands in an MHC-independent fashion expressed by cancer cells of diverse origins \(^3\) \(^7\) \(^8\). Because the ligands for the NKG2D receptor are absent or expressed at very low levels in normal tissues \(^1\) \(^9\), specificity for tumors is considered to be high. In agreement, we previously demonstrated no recognition of normal peripheral blood mononuclear cells nor healthy bone marrow from healthy patients by CYAD-01 \textit{in vitro} \(^10\). Preclinical studies have shown that CYAD-01 mediates potent anti-tumor activity against both hematological and solid tumors without the requirement of prior lymphodepleting chemotherapy \(^3\) \(^6\). Single intravenous administration of low dose autologous CYAD-01 (maximum 3x10\(^7\) flat dose),
without prior chemotherapy, has been tested in a completed Phase I study (NCT02203825) which evaluated 12 patients with relapsed/refractory AML (r/r AML), myelodysplastic syndrome (MDS) or multiple myeloma (MM) \textsuperscript{11}. The preliminary data obtained suggested encouraging signs of activity with one AML patient treated at the \(3 \times 10^7\) CYAD-01 dose-level having hematologic improvement for three months post-CYAD-01 treatment \textsuperscript{11}. Building on these initial results and preclinical data supporting multiple CYAD-01 infusions are necessary for disease eradication, we initiated the THINK study (ClinicalTrials.gov number: NCT03018405) evaluating the safety and clinical activity of multiple treatment administrations of CYAD-01 in different solid and hematological indications \textsuperscript{6}.

We report here the case of a 52 years old male with r/r AML. The patient had +8/del(7)(q22q36), \textit{FLT3}/\textit{NPM1} wild-type AML that was primary refractory to induction with 7+3 with daunorubicin and cytarabine. The patient subsequently received salvage chemotherapy with cladribine, cytarabine, G-CSF and mitoxantrone (CLAG-M) and achieved CR\textsuperscript{1} followed by two cycles of CLA consolidation. Following a 7 months remission where allogeneic hematopoietic stem cell transplantation (allo-HSCT) was delayed to allow for pulmonary function test recovery, the patient had recurrent cytopenias and bone marrow (BM) biopsy confirmed relapsed disease with hypocellularity, 7\% blasts in numerous clusters and dysmegakaryopoiesis (Fig. 1A and 1B). The patient developed severe constitutional symptoms with fatigue, anorexia, body pain and drenching night sweats. Given no standard treatment options, the patient enrolled in the THINK trial and underwent apheresis followed by CYAD-01 infusions at the initial dose level of \(3 \times 10^8\) cells (flat dose)/injection every 2 weeks for 3 administrations without any lymphodepleting chemotherapy \textsuperscript{6}. CYAD-01 was well tolerated with non-related grade 1 adverse events without cytokine release syndrome (CRS) or neurotoxic effects. BM evaluation at day +28 following 2 CYAD-01 infusions

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showed morphologic leukemia-free state (MLFS, 2% blasts) with normocellularity and trilineage hematopoiesis, which was confirmed at day +56 BM with del(7q) by FISH at 7% (Fig. 1C). Serial next-generation sequencing (NGS) of BM-MNCs showed persistent \textit{DNMT3A} R882H mutation, but also identified a new \textit{IDH2} R172K mutation. The patient had resolution of symptoms with improved hematopoiesis, which continued to improve until the patient was treated with an allo-HSCT on day +97 post CYAD-01 (Fig. 1D). At disease evaluation 100 days post allo-HSCT (day +197 post CYAD-01), the patient achieved a complete molecular remission by serial NGS with 100% donor chimerism (Fig. 1D). To date, the patient has maintained a CR 6 months from allo-HSCT and 9 months following initial CYAD-01 infusion. Fig. 2A shows timeline of the patient’s history of treatments and responses.

Molecular analysis indicated a low level of CYAD-01 cells in the periphery until day +4 post injection. The patient CYAD-01 product primarily consisted of effector-memory phenotype \textit{CD8}^+ T-cells with robust target cell-directed cytotoxicity and interferon (IFN)-\textit{γ} secretion (Fig. S1A and S1B). Immunohistochemistry (IHC) analysis on the baseline BM taken showed positive staining of foci of neoplastic cells for all NKG2DL assessed (Fig S2), with the strongest expression for MICA/MICB (Fig. 2B). Of note, there was no NKG2DL expression on the non-neoplastic BM cells. Finally, it is important to mention no detectable level of the transgene was evidenced at day +1, +4 and +32 post-injection, using a Vector copy number (VCN) qPCR-based assay, suggesting poor in vivo expansion and persistence of the injected cell product.

CAR therapy has brought forth a paradigm change in the management of refractory chemo-resistant B-cell leukemia. However, the choice of target in AML has been challenging due
to their normal expression on hematopoietic stem cells leading to potential irreversible hematopoietic toxicity\textsuperscript{13}. Thus, current CAR-T strategies in AML either require a backup allo-HSCT or unproven shut-off mechanisms\textsuperscript{14}. In contrast, NKG2DL are upregulated in transformed cells with minimal expression in normal tissues\textsuperscript{1}. This was confirmed by the absence of significant adverse events of CYAD-01 in this AML patient and no Grade 3 or above adverse events related to treatment in any of the AML patients enrolled in the THINK trial up to now. This is of critical clinical importance given CD19 specific CAR-T cell therapies with grade 5 toxicities including CRS, neurotoxicity and on-target off-tumor toxicity against normal cells\textsuperscript{15}. The low toxicity, with clinical activity, observed in this patient is most probably related to multiple factors: (i) CYAD-01 cells demonstrate only short-term persistence and low proliferation post-infusion, (ii) the chimeric CYAD-01 construct consists entirely of human sequences, with an entirely native extracellular domain not expected to induce the anaphylaxis reactions observed with other CAR-Ts using scFv from murine origin\textsuperscript{16}, and (iii) the absence of previous lymphodepleting chemotherapy.

While reduction of blasts occurred in AML patients treated with LeY-specific CAR-Ts was also previously reported, the clinical response could not be entirely attributed to the activity of the CAR-T since these patients were in morphological CR after reinduction with fludarabine prior to CAR-T administration\textsuperscript{17}. In the only published r/r AML case report evaluating the CD33-specific CAR-Ts without conditioning therapy, a 2-weeks transient decrease in blast count was reported in the context of grade 4 chills, fevers and transient hyperbilirubinemia, but with a rapid disease progression, suggesting tumor escape mechanism\textsuperscript{18}. Of clinical interest in the setting of MLFS and blood counts approaching complete remission at the time of allo-HSCT, the patient had identification of a \textit{IDH2} mutation that was not present prior to treatment. This suggests that there can be heterogeneity of NKG2D ligand expression that is
dependent on the underlying molecular architecture. This is supported by a recent study in \textit{IDH} mutant gliomas where NKG2D ligands were down-regulated \textsuperscript{19}. Future study is required to further investigate molecular drivers of NKG2D ligand expression in AML in order to further augment efficacy of CYAD-01. In summary, we report the first objective response to CAR-T in r/r AML using CYAD-01 without preconditioning chemotherapy and with no significant toxicities, highlighting the potential of targeting NKG2DL in AML.

\textbf{Conflict of Interest}

CL, EB, SA, BV, DEG and FFL are employed by Celyad SA. The THINK clinical trial is sponsored by Celyad SA.

\textbf{References}


Figure Legends

Figure 1. Relapsed AML patient treated with CYAD-01 CAR-T therapy. CD34 IHC of the core biopsy shows clusters of blasts (~7%) consistent with relapsed disease (Panel A). Prominent dysmegakaryopoiesis was also noted (Panel B). BM biopsy at day +56 following 3 infusions of CYAD-01 showed normal trilineage hematopoiesis with CD34 IHC showing no increase in blasts (Panel C). Patient also with significant improvement of hematopoiesis as transfusion independent with marked increase of hemoglobin and platelet count (Panel D).

Fig. 2: Treatment Course and immunohistochemistry analysis of NKG2D ligand expression in bone marrow sample taken before first CYAD-01 injection. Timeline of history of treatments and responses is shown (A). BM biopsy of patient prior to CYAD-01 treatment was subject to a range of IHC staining protocols specific for the following NKG2DL: MICA/MICB, ULBP1, ULBP2/5/6, ULBP3. In addition, a sample of each was prepared applying standard hematoxylin and eosin (H&E) staining (data not shown). All samples were examined and graded by light microscopy. 100 presumptive neoplastic cells were counted and analysed, in terms of staining characteristics, using a “H-score” approach. The intensity of membranous staining was graded for each cell, as follows: 0 – no staining; 1 – minimal staining (each positive cell scored “1”); 2 – mild staining (each positive cell scored “2”); 3 – moderate/marked staining (each positive cell scored “3”). The percentage of positive cells for each intensity staining and each ligand is represented in (B). Representative staining of MICA/B is shown in (C).
Supplementary information for:

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*These authors contributed equally to this work.
Fig. S1: Patient’s CYAD-01 phenotype and *in vitro* functionality. (A) A sample of the infusion product was analyzed for cell subset composition and memory phenotype. CD3+ viable singlet cells showed a high CD8 preponderance with both CD4 and CD8 subsets possessing a majority of CD62L$^{lo}$ phenotype. As shown in the CD8 subset, both CD62L$^{hi}$ and CD62L$^{lo}$ populations were CD95$^+$ and CD28$^{lo}$. Together this suggests the infusion product to be composed primarily of CD8$^+$ T cells that possess an effector memory phenotype. 88% of the CD4$^+$ T cells and 94% of the CD8$^+$ T cells were NKG2D+. (B) Patient’s CYAD-01 product was incubated in the presence or absence of NKG2D blocking antibody (CD314 Ab) (light and dark grey bars, respectively) with PANC-1 and K562 cancer cells (at a 1:1 ratio). After 24h of incubation, supernatants were harvested and analyzed for IFN-$\gamma$ secretion. Dotted line represents the limit of detection (LOD). Each bar represents the mean and SD of one experiment conducted at least in duplicate, ****: p < 0.001. (C) Patient’s CYAD-01 cells were cultured at a 1:1 ratio with PANC-1 cells in the presence or absence of CD314 blocking Ab. After a 20h
incubation the CYAD-01 cells were washed away and the remaining PANC-1 cells stained with AlamarBlue to quantify the remaining proliferating PANC-1 cells. Each bar represents one experiment conducted in triplicate with the corresponding SD, *: p < 0.05.
Fig. S2: Immunohistochemistry analysis of NKG2D ligand expression in bone marrow sample taken before first CYAD-01 injection. One BM biopsy was subject to a range of IHC staining protocols specific for the following NKG2DL: MICA/MICB, ULBP1, ULBP2/5/6, ULBP3. In addition, a sample of each was prepared applying standard hematoxylin and eosin (H&E) staining (data not shown). All samples were examined and graded by light microscopy. Representative stainings of ULBP1 (A), ULBP2/5/6 (B) and ULBP3 (C). It has to be noted that ULBP2/5/6 are predominantly stained in the nucleus and that ULBP1, ULBP3 and MICA/B (Fig. 2B) display cytoplasmic staining as well.
Supplementary Methods

Study THINK design

The THINK (Therapeutic Immunotherapy with NKR-2) trial is an open-label Phase I study which primarily aims to assess the safety and clinical activity of the CYAD-01 treatment administered three times at 2 weeks intervals between each administration without prior lymphodepleting chemotherapy in patients with refractory or relapsing malignancies, including patients with metastatic or locally advanced colorectal cancer, urothelial carcinoma, triple-negative breast cancer, pancreatic cancer, recurrent epithelial ovarian and fallopian tube carcinoma, AML/MDS or MM. The study is split into two segments; a dose escalation segment evaluating three dose-levels (3x10^8, 1x10^9 and 3x10^9 cells/injection) to determine the recommended dose of CYAD-01 cells and an expansion phase to investigate the clinical activity across multiple tumor indications while extending the safety study.

Manufacture of cell products

CYAD-01 (previously known as NKR-2) refers to the viable cell population obtained after retroviral transduction of autologous T-cells with the NKG2D-based CAR. CYAD-01 will be supplied cryopreserved in bags containing a T-cell dose in accordance with the dose-level which is to be administered.

Characterization of the patient CYAD-01

CYAD-01 identity (% NKG2D on CD4+, CD4+CD8+ and CD8+ T cells), purity (% viable CD3+) and viability (with 7AAD dye) are assessed by flow cytometry. Cell yield is assessed by cell counting (excluding Trypan blue). In vitro product functionality/potency is evaluated by assessment of IFN-γ secretion via ELISA and metabolic activity of tumor cells via Alamar Blue assay upon co-culture of CYAD-01 with NKG2D ligand-expressing tumor cells (Panc-1 cells
and/or K562). NKR-2 microbiological safety was confirmed by absence of microbiological
growth, assessed by BactAlert, absence of mycoplasms, assessed by qPCR based MycoTool
assay, and compliant endotoxin level (≤8.67 EU/ml), assessed by PTS EndoSafe LAL assay.
Patient CYAD-01 met the product specifications. CYAD-01 phenotype identity (90% NKG2D+), purity (95% CD3+), viability (86%) and cell yield (300 x 10^6 cells), evidenced a
viable, highly pure CD3+, NKG2D+ product. Viral vector safety was evaluated by Vector copy
number (VCN) and Replication copy number (RCR) qPCR-based assays. In vitro product
functionality/potency was confirmed by IFN-γ secretion and efficient cytotoxicity effect in
response to NKG2DL on tumor cells.