ARTICLE

ACUTE MYELOID LEUKEMIA

Downregulation of MICA/MICB improves cell persistence and clinical activity of NKG2DL CAR T-cells in patients with relapsed or refractory acute myeloid leukemia or myelodysplastic neoplasia

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The NKG2D receptor binds eight ligands (NKG2DL) overexpressed in a wide range of malignancies, but largely absent on non-neoplastic cells. Initial clinical evaluation of NKG2DL chimeric antigen receptor (CAR) T-cells (CYAD-01) in patients with relapsed or refractory (r/r) acute myeloid leukemia (AML) or myelodysplastic neoplasia (MDS) demonstrated low durability of responses and short cell persistence. Two Phase I trials were initiated to evaluate the effect of lymphodepletion prior to a single CAR T-cell infusion in a similar r/r AML/MDS patient population. The DEPLETHINK trial (NCT03466320) evaluated CYAD-01 while the CYCLE-1 trial (NCT04167696) evaluated a next-generation NKG2DL CAR, CYAD-02, where the two main NKG2D ligands MICA and B are downregulated, to increase CAR T-cell persistence. Seventeen and twelve patients were treated in the DEPLETHINK and CYCLE-1 trials, and confirmed the good tolerability of both products with cytokine release syndrome (CRS) grade 3 or 4 reported in 25% and 33.3% of patients, respectively. CYAD-02 presented an higher engraftment and an improved clinical activity (17% objective response rate) compared to CYAD-01 (no objective response). Altogether, our data provide proof of principle that knock-down of MICA/B can enhance CAR T-cell persistence and efficacy while maintaining a good safety profile.

Leukemia (2025) 39:2907–2915; https://doi.org/10.1038/s41375-025-02767-4

INTRODUCTION

During the past decade several chimeric antigen receptor (CAR) T-cell therapies for patients with B-cell lymphoma, B-cell leukemia, or multiple myeloma, have been approved by authorities from several countries, based on the observed impressive overall response rates and durable remissions in these patient populations [1–3]. However, efforts are still needed to generalize CAR T-cell therapy beyond B-cell and plasma cell malignancies. Acute myeloid leukemia/myelodysplastic neoplasia (AML/MDS) is characterized by a high genetic and epigenetic heterogeneity [4] and a highly immunosuppressive microenvironment (TME) that facilitates tumor escape mechanisms leading to disease relapse [5]. Therefore, despite remarkable progress, AML remains a significant unmet need, as evidenced by very poor outcomes of relapsed or refractory (r/r) AML/MDS patients [6]. Currently, allogeneic hematopoietic stem cell transplantation (allo-SCT), when feasible, is the only potentially curative strategy for most patients with r/r AML/MDS [7]. In this context, the development of alternative treatments such as CAR T-cell therapies is urgently needed. However, in addition to the challenges listed above, the lack of universal surface leukemic blast-specific antigen targets for MDS/AML, leading to important toxicities mediated by expression on normal hematopoietic stem cells or mature myeloid populations, but also antigen heterogeneity and target antigen loss through clonal selection are further obstacles to overcome in the development of single safe and efficient CAR T-cell approaches [5, 8, 9].

The natural killer group 2D (NKG2D) receptor is an activating receptor, usually expressed on natural killer (NK) cells, that binds eight stress-induced ligands belonging to two families, the major histocompatibility complex class I chain related proteins A (MICA) and B (MICB), and the structurally diverse unique long 16 binding proteins, i.e., UL16-binding proteins 1 to 6 (ULBP1-6) [10]. These ligands (NKG2DL) are overexpressed on tumor cells from a large majority of solid and hematological cancers, including AML/MDS, while their expression on healthy non-neoplastic cells (including

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Received: 23 June 2025 Revised: 16 August 2025 Accepted: 8 September 2025
Published online: 15 September 2025

stem cells) remains largely absent or undetectable, making them promising targets for CAR T-cell approaches [11].

We designed an autologous CAR T-cell product engineered to express a NKG2DL CAR consisting of the full-length human NKG2D receptor fused with the human CD3ζ signaling domain which interacts with the endogenous adaptor molecule DNAX-activating protein of 10 kDa (DAP10) to mediate signal transduction upon ligand recognition [12]. This product, CYAD-01, was evaluated in several Phase I studies in AML/MDS, multiple myeloma or solid cancers and demonstrated early signs of activity (25% objective response rate in AML/MDS in the THINK trial) [13–15] and a good tolerability, but a short duration of responses was observed and was correlated with short-term persistence and low expansion of the CAR T-cells post-infusion in the peripheral blood [13, 14].

CYAD-01 was initially tested using a multiple-injection schedule without prior preconditioning [14, 16]. To explore the traditional CAR-T therapy strategy, the DEPLETHINK trial was launched. This trial incorporated a lymphodepleting chemotherapy (LD) regimen, which is considered essential for enhancing CAR T-cell therapy by promoting the expansion and persistence of CAR T-cells following infusion.

We previously discovered that MICA and MICB were transiently expressed on the surface of the CAR T-cells themselves, potentially leading to self-recognition and fratricide, which could impair their activity and persistence [17]. To avoid this, the manufacturing process of CYAD-01 had been previously modified to include a NKG2D-blocking antibody ('mAb' process) to increase manufacturing yield [14]. However, we hypothesized that the transient expression of MICA/MICB on CYAD-01 could still, at least partially, contribute to its limited persistence in vivo, i.e., post infusion. This phenomenon is similar to that observed in other CAR T-cell therapies targeting antigens like CD5 or CD7, which are shared between normal and malignant T-cells [18].

To address this we developed a microRNA (miRNA)-based short hairpin RNA (shRNA) to genetically modify CAR T-cells by downregulating the specific target genes [19]. This approach had already demonstrated safety and efficacy in a first-in-human clinical trial [20]. Using this platform, we developed a single miRNA-based shRNA capable of simultaneously silencing both MICA and MICB, thereby preventing NKG2DL CAR T-cells from fratricide. Co-expression of this miRNA-based shRNA with the NKG2DL CAR gave rise to a next-generation CAR T-cell product, termed CYAD-02.

The present publication covers clinical data of the first-in-human Phase I clinical trial of CYAD-02 in a r/r AML/MDS patient population (CYCLE-1, NCT04167696), as compared to clinical data from the Phase I DEPLETHINK trial (NCT03466320), evaluating CYAD-01 with a similar treatment schedule and in a similar patient population.

METHODS

CYAD-01 and CYAD-02 constructs

The NKG2DL CAR construct contains the full-length NKG2D fused to the intracellular CD3ζ. Primary human T-cells transduced with this vector were termed CYAD-01. For CYAD-02, the construct co-expressed the NKG2DL CAR, a truncated form of CD19 (tCD19) reporter gene and a miRNA-based shRNA for human MICA/B (shRNA guide strand: 5′-TCTCTGTTTCATAGGTCAGG-3′).

Trial design

DEPLETHINK (CYAD-N2T-005) is a Phase I, open-label, clinical trial conducted at 3 sites in Belgium (EudraCT 2018-000205-22) and 4 sites in the United States of America (NCT03466320) and evaluating CYAD-01 in relapsed or refractory (r/r) AML/MDS patients. CYCLE-1 (CYAD-02-001) is a Phase I, open-label, clinical trial conducted at 3 sites in Belgium (EudraCT 2019-001816-46) and 1 site in the United States of America (NCT04167696) and evaluating CYAD-02 in r/r AML/MDS patients.

For both DEPLETHINK and CYCLE-1, the dose-escalation segment evaluated three dose-levels: 1×10^8 (DL1), 3×10^8 (DL2) and 1×10^9 (DL3) total cells per infusion (adjusted per body weight for patients weighing ≤65 kg i.e., at 1.5×10^6, 4.6×10^6 and 1.5×10^7 cells/kg, respectively), using a Fibonacci 3 + 3 design to determine the recommended dose of CYAD-01 (DEPLETHINK) or CYAD-02 (CYCLE-1) when administered as a single infusion on Day (D) 1 after LD (cyclophosphamide 300 mg/m²/day and fludarabine 30 mg/m²/day, for 3 days administered from D-5 to D-3). Patients not in progressive disease after first infusion were allowed to receive a consolidation cycle with three additional CYAD-01/02 infusions at a 2-week interval without additional preconditioning, and at the same dose as for the first infusion, as soon as the patient did not present any toxicity related to the first CAR T-cell infusion and that the peripheral blood levels of CAR T-cell was below the detection level of the quantitative polymerase chain reaction assay before the initiation of the consolidation cycle.

Eligibility criteria

Patients were 18 years of age and older with an Eastern Cooperative Oncology Group (ECOG) performance status ≤ 2. Eligible patients had either (i) a confirmed r/r acute AML (i.e., ≥ 5% blasts in bone marrow or in peripheral blood) with revised European LeukemiaNet (ELN) 2017 risk stratification [21] for favorable, intermediate or adverse groups, after at least one prior therapy or (ii) a confirmed MDS as defined by revised International Prognostic Scoring System criteria [22] for intermediate, high-risk or very high-risk disease or MDS with TP53 mutation as detected by next-generation sequencing, after failure of prior treatment with at least 4 cycles of azacitidine or decitabine. The absolute peripheral blast count at baseline should be <15,000/µL. Additional baseline assessments required for eligibility, as assessed by standard laboratory criteria and spirometry, included adequate hepatic and renal functions, a left ventricular ejection fraction of ≥ 40% and a forced expiratory volume in the first second (FEV-1)/forced vital capacity (FVC) ≥ 0.7 with FEV-1 ≥ 50% predicted. Serious uncontrolled medical disorders, persistent toxicities greater than or equal to Common Terminology Criteria for Adverse Events (CTCAE) grade 2 caused by previous cancer therapy, active infections, or any cancer treatment within 2 weeks of the planned date of apheresis, or prior allogeneic SCT or donor lymphocyte infusions within 3 months before the planned day of the apheresis were the main exclusion criteria.

Endpoints and procedures

The primary endpoint of these open-label phase I dose escalation studies was the occurrence of dose-limiting toxicities (DLTs) at any time after initiation of the CYAD-01 or CYAD-02 infusion up to D36 or D85 (in case of consolidation cycle). DLT was defined as a toxicity at least possibly related to the CYAD-01 or CYAD-02 treatment including (1) any Common Terminology Criteria for Adverse Events (CTCAE) or CRS/CAR T-cell-associated neurotoxicity (CRES) grade 5 toxicity not due to the underlying malignancy, (2) any CTCAE Grade 3 or higher allergic reactions related to the CAR T-cell infusion that cannot be controlled to ≤Grade 1 within 24 h of treatment with appropriate treatment, (3) any CTCAE Grade 3 or higher CYAD-01 or CYAD-02-related toxicity that could lead to irreversible damage of a vital organ, (4) any CTCAE Grade 4 CYAD-01 or CYAD-02-related neutropenia or thrombocytopenia not associated with disseminated intravascular coagulation that cannot be controlled to ≤Grade 2 or to patient baseline hematological values within 42 days with appropriate treatment, or (5) any Grade 4 CRS and/or CRES of any duration.

Disease assessment in the peripheral blood (PB), in the bone marrow (BM) biopsies or aspirates was performed at baseline, and at pre-specified timepoints after the CYAD-01 or CYAD-02 infusion. Responses were assessed by the investigators according to the Revised Recommendations of the International Working Group (IWG) for Diagnosis, Standardization of Response Criteria [23] for AML patients, or IWG 2006 Uniform Response Criteria for patients with MDS [24]. Severity of AEs was graded according to the National Cancer Institute (NCI)'s CTCAE (version 5.0) and evaluated at each visit. Severity of CRS and CRES was graded by the guidelines from the CAR-T-cell therapy-associated TOXicity (CARTOX) working group, at that time the internationally used score for CAR-T associated toxicities [25]. The updated ASTCT grading system to evaluate CRS and Immune effector cell-associated neurotoxicity syndrome was released after the initiation of both trials, hence was not use to maintain harmonization of toxicity assessment and management between patients [26].

Table 1. Patients' main characteristics per cohort in the DELETHINK and CYCLE-1 trials.

Characteristics	DEPLETHINK				CYCLE-1			
	DL1 ($N = 6$)	DL2 ($N = 6$)	DL3 ($N = 5$)	Total ($N = 17$)	DL1 ($N = 3$)	DL2 ($N = 3$)	DL3 ($N = 6$)	Total ($N = 12$)
Age, median (range), (years)	61 (50–73)	70 (58–75)	68 (50–72)	68 (50–75)	67 (66–79)	74 (41–74)	70 (60–78)	70 (41-79)
Gender, %, (Male/Female)	67/33	67/33	60/40	47/52	33/67	100/0	50/50	58/42
ECOG score at screening, %, 0/1/2	83/17/0	17/83/0	0/80/20	35/59/6	0/100/0	33/67/0	67/17/17	42/50/8
Tumor type								
r/r Acute myeloid leukemia, *n* (%)	4 (67)	5 (83)	5 (100)	14 (86)	1 (33)	2 (67)	3 (50)	6 (50)
r/r Myelodysplastic syndrome, *n* (%)	2 (33)	1 (17)	0	3 (14)	2 (67)	1 (33)	3 (50)	6 (50)
ELN 2017/R-IPSS risk stratification								
Favorable (AML) / Intermediate (MDS)	0 / 0	0 / 0	1 / 0	1 / 0	0 / 0	0 / 0	1 / 0	1 / 0
Intermediate (AML) / High-Risk (MDS)	4 / 0	1 / 1	2 / 0	7 / 1	0 / 0	0 / 1	0 / 1	0 / 2
Adverse (AML) / Very High-Risk (MDS)	0 / 2	4 / 0	1 / 0	5 / 2	1 / 2	2 / 0	2 / 2	5 / 4
Bone marrow blasts (%) median (range)	30 (6–48)	26 (3–42)	29 (23–63)	26 (3–63)	17 (14–63)	25 (4–28)	6.5 (2–81)	13.5 (2–81)
Peripheral blood blasts (%) median (range)	3.2 (0–43)	1.1 (0–42)	24.6 (0–36)	4 (0–43)	11.2 (0–22.1)	1.0 (0–21)	0.5 (0–4.6)	1.0 (0–22.1)
Number of prior lines of therapy, median (range)	3 (1–4)	1 (1–3)	2 (1–6)	2 (1–6)	1 (1–1)	1 (1–6)	1 (1–2)	1 (1–6)

AML acute myeloid leukemia, *DL* dose-level, *ECOG* Eastern Cooperative Oncology Group, *r/r* relapsed or refractory, *ELN* European Leukemia Network, *IPSS* International Prognostic Scoring System, *MDS* myelodysplastic neoplasia, *r/r* relapsed or refractory.

All statistical analyses for these open-label phase I dose escalation studies were primarily descriptive. For descriptive statistics and result representations, GraphPad Prism 9 was used.

Ethics approval and consent to participate

The protocols, and patient informed consents (original versions and all subsequent amendments), were approved by the respective institutional review board or independent ethics committee before trial initiation at each site. DEPLETHINK trial was approved by the Central Ethical Committee of the University Hospital Ghent (UZ Ghent) and all respective Belgian independent ethics committees (04 June 2018) and by the US institutional review boards at each site (NYU: 30 July 2018; UCD: 23 July 2018; Emory University: 05 September 2019; Moffitt: 11 June 2019), before study initiation. CYCLE-1 trial was approved by the Central Ethical Committee of the University Hospital Leuven (UZ Leuven) and all respective Belgian independent ethics committees (21 October 2019) and by the KUMC US institutional review board (21 November 2019), before study initiation.

The trials were conducted in accordance with the Declaration of Helsinki and adhered to the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use guideline for Good Clinical Practice. All patients provided written informed consent before registration in the trials.

Cellular kinetics

CYAD-01 and CYAD-02 cellular kinetic analysis in patient's samples was determined by digital droplet polymerase chain reaction (ddPCR) on genomic DNA extracted from peripheral blood mononuclear cells isolated from whole blood collected at pre-specified timepoints (at baseline, then on D-5, D1, D3, D5, D8, D11, D15, D22, D29, D36, D85 and Month (M)6, M9, M12, M18 and M24 after the first infusion, and in case of a consolidation cycle, also the day of infusions and a week later). Transgene and housekeeping genes expression were quantified using BioRad ddPCR system. Cellular kinetics data are expressed as number of copies of the transgene/µg of genomic DNA. For descriptive statistics and result representations, GraphPad Prism 10 was used.

RESULTS

Clinical evaluation of a single infusion CYAD-01 with a prior preconditioning in r/r AML/MDS patients

The open-label Phase 1 DEPLETHINK trial (NCT03466320, EudraCT NCT03466320) was designed to evaluate the safety and clinical efficacy of CYAD-01 after LD in adult patients with refractory or relapsed (r/r) AML or MDS. Patients received preconditioning for 3 days (cyclophosphamide 300 mg/m^2/day and fludarabine 30 mg/m^2/day, 'CyFlu') followed by a single CYAD-01 infusion at one of the three different dose-levels (DL): 1×10^8, 3×10^8 and 1×10^9 total cells/infusion.

Between 23 October 2018 and 09 July 2020, seventeen patients were enrolled across the 3 DLs in the DEPLETHINK trial (Fig. S1). Patients main baseline characteristics are shown in Table 1. Patients were 68 years old on average with 47% male patients and 52% female patients. At screening, 86% of the patients had r/r AML and 14% had r/r MDS, with a median of 2 prior lines of treatment.

Clinical evaluation of a single infusion CYAD-02, expressing a miRNA-based construct against MICA/B, with a prior preconditioning in r/r AML/MDS patients

To prevent fratricide caused by the expression of NKG2DL on the surface of NKG2DL CAR T-cells, we engineered a retroviral vector encoding a miRNA-based shRNA designed to target homologous regions shared by MICA and MICB, together with the NKG2DL CAR and a truncated CD19 (tCD19) reporter gene (Fig. 1A). Primary human T-cells transduced with this construct were termed CYAD-02.

CYAD-02 was evaluated in the open-label Phase 1 CYCLE-1 trial (NCT04167696, EudraCT 2019-001816-46), conducted in adult patients with r/r AML or MDS. The trial employed a study design, treatment schedule, and patient population similar to those used

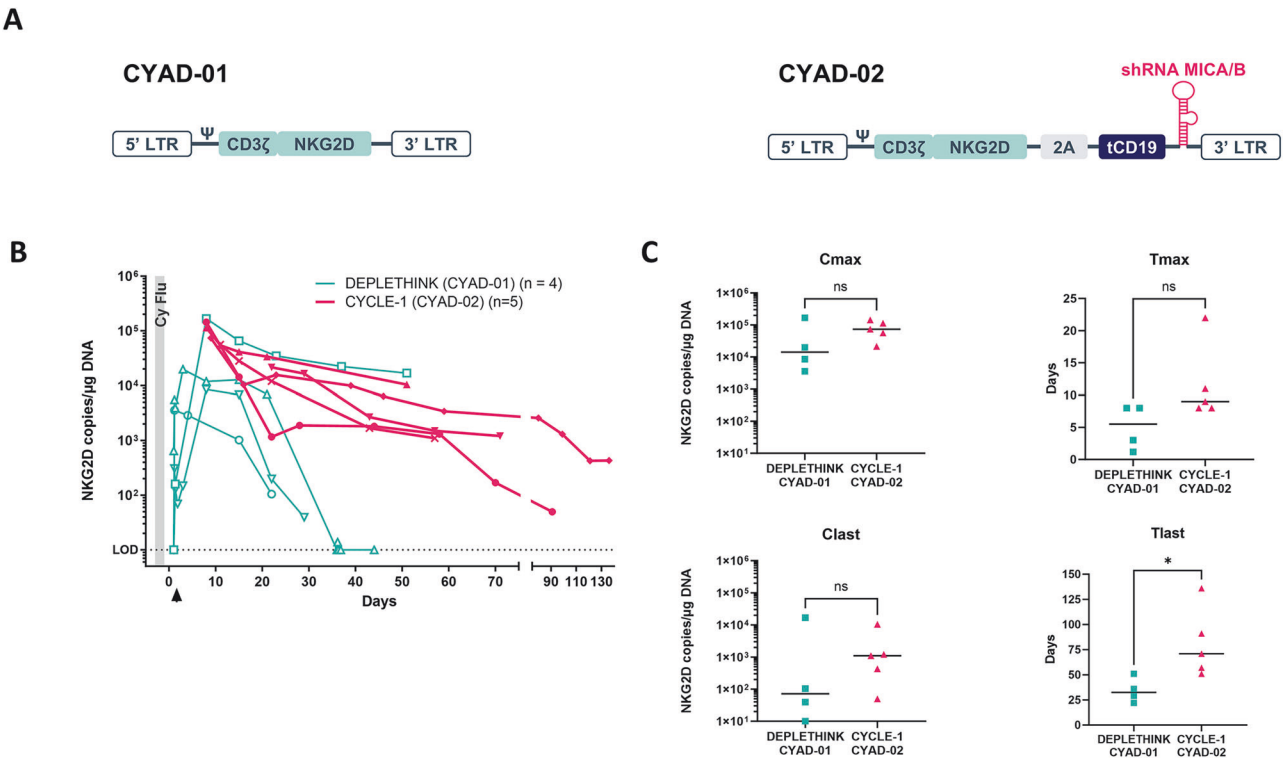

Fig. 1 miRNA-based shRNA against MICA/B increases persistence of NKG2D CAR T cells. A Schematic representations of the CYAD-01 and CYAD-02 constructs. **B** Cellular kinetics of CYAD-01 and CYAD-02 at highest dose administered (1×10^9 cells/inf.) in the CYCLE-1 and DEPLETHINK studies. Cell kinetics were determined by ddPCR using gDNA isolated from blood collected at pre-specified timepoints. Values below the limit of detection (LOD) of the assay are set at LOD (depicted as a dotted line). Gray shadowed area indicates days of CyFlu preconditioning administration. **C** Kinetics parameters. Cmax: highest number of transgene copies/µg of DNA observed in peripheral blood after first treatment administration at Tmax. Clast: last detectable measure of transgene copies/µg of DNA observed in peripheral blood after first treatment administration at Tlast. *p value of < 0.05. All parameters were calculated before the initiation of the consolidation cycle, if applicable. One patient in CYCLE-1 (#AML 12) is not shown as he received a delayed infusion of CAR T cells 10 days post CyFlu administration, which negatively impacted the peak engraftment of CYAD-02 compared to other patients at the same dose. Only patients who received the optimized manufacturing process are included.

in the DEPLETHINK trial, enabling a direct comparison between the two product candidates.

Between 07 January 2020 and 28 July 2021, twelve patients were enrolled across the 3 DLs in CYCLE-1 trial (Fig. S1). Patients were 70 years old on average with 58% male patients and 42% female patients. At screening, 50% of the patients had r/r AML and 50% had r/r MDS, with a median of 1 prior line of treatment (Table 1).

Safety profile of CYAD-01 and CYAD-02

During the DEPLETHINK trial, a manufacturing process amendment was implemented—following approval by the US FDA and Belgian FAMHP—to introduce an optimized process, referred to as "OptimAb", for the production of CYAD-01. This modification aimed mostly to improve cell phenotype while maintaining the manufacturing yield (see Supplementary Methods). A total of seven patients received CYAD-01 manufactured using this optimized process in the DEPLETHINK trial (Fig. S1).

In contrast, CYAD-02 was developed from the beginning using the OptimAb process, and the CYCLE-1 trial commenced after its implementation. To enable a fair comparison between CYAD-01 and CYAD-02, and to ensure that the only variable distinguishing the two products is the inclusion of the miRNA-based shRNA targeting MICA/B in CYAD-02, only patients treated with CYAD-01 produced via the "OptimAb" process manufactured-CYAD-01 were included in the safety, activity and cell kinetics analyses of the present publication.

Safety profile for both products in DEPLETHINK (only OptimAb patients) and CYCLE-1 is presented in Table 2 and in Table S1. Overall, no dose-limiting toxicity (DLT) nor treatment-related death were reported after CYAD-01 treatment in the DEPLETHINK trial. All patients (100%) experienced adverse events (AEs). One unrelated grade 5 AE (disease progression) was reported in a patient at DL2. Grade 4 AEs (reported in 5 patients) included lymphocyte, neutrophil or platelet count decreased, anemia or thrombocytopenia. All patients (100%) experienced treatment-related AEs with CRS as the most commonly reported treatment-related AE (85.7% of patients), followed by pyrexia (57.1% of patients), chills (28.6% of patients), abnormal breath sounds, hypotension, decreased lymphocyte count, neurotoxicity, decreased oxygen saturation, pulmonary oedema, decreased appetite, acute respiratory failure and diarrhea (each 14.3% of patients). 25% of patients had treatment-related grade 3 AEs. Treatment-related serious adverse events (SAEs) were reported for 2 patients (one patient with grade 2 CRS and neurotoxicity at DL2 and one patient with grade 3 CRS at DL3). For completeness, the data for the patients in DEPLETHINK who were treated with the prior process can be found in the Supplementary data (Table S2).

Similarly, no CRES nor treatment-related death were reported with CYAD-02 in CYCLE-1. All patients (100%) experienced AEs. Two patients had grade 5 unrelated AEs reported: one venoocclusive disease in a patient at DL2 and one neutropenic sepsis in a patient at DL3. Grade 4 AEs were reported in eight patients and included infusion-related reaction, CRS, neutrophil, platelet or white blood cell decreased, neutropenia, thrombocytopenia, pancytopenia, peripheral ischemia and pneumonia pseudomonal. All patients (100%) experienced treatment-related AEs with CRS as

Table 2. Treatment adverse events per grade reported in the DEPLETHINK and CYCLE-1 trials.

AE of interest	Nb of patients presenting adverse events (AE) of interest (highest grade)											
	DEPLETHINK ($N = 7$*)						CYCLE-1 ($N = 12$)					
	1	2	3	4	5	All	1	2	3	4	5	All
Any AE	7 (100%)	7 (100%)	7 (100%)	5 (71%)	1 (14%)	7 (100%)	12 (100%)	12 (100%)	12 (100%)	8 (67%)	2 (17%)	12 (100%)
AE related to CYAD-01 / 02	5 (71%)	6 (86%)	4 (57%)	–	–	7 (100%)	8 (67%)	7 (58%)	5 (42%)	4 (33%)	–	12 (100%)
SAE related to CYAD-01 / 02	–	1 (14%)	1 (14%)	–	–	2 (28%)	–	–	3 (25%)	2 (17%)	–	4 (33%)
CRS[a]	3 (43%)	3 (43%)	1 (14%)	–	–	6 (86%)	6 (50%)	4 (33%)	3 (25%)	1 (8%)	–	11 (92%)
CRES[a]	–	1 (14%)	–	–	–	1 (14%)	–	–	–	–	–	–
Infection[b]	1 (14%)	2 (28%)	1 (14%)	–	–	3 (42%)	–	3 (25%)	1 (8%)	1 (8%)	–	4 (33%)
Pulmonary disorders[c]	–	2 (28%)	1 (14%)	–	–	2 (28%)	–	–	–	-	–	–
Infusion reaction	–	–	–	–	–	–	–	–	–	1 (8%)	–	1 (8%)

AE adverse event, *CRES* CAR-T cell-related encephalopathy syndrome, *CRS* cytokine release syndrome, *SAE* serous adverse event.
* Only patients who received the optimized manufacturing process.
[a]As per CAR-T-cell-therapy-associated TOXicity (CARTOX) Working Group criteria from Neelapu (2018) Nat Rev Clin Oncol. 15(1):47–62.
[b]Bacterial, viral and fungal infections within 28 days of last CAR T-cell infusion (all relatedness to CYAD-01/02).
[c]Including acute respiratory failure, pulmonary oedema and non-cardiogenic pulmonary oedema (all relatedness to CYAD-01/02).

the most commonly reported AE (91.7% of patients), followed by white blood cell count decreased, fatigue, disseminated intravascular coagulation and febrile neutropenia (each 16.7% of patients). 33.3% of patients had grade 3 or 4 CRS across the 3 dose levels. Treatment-related SAEs were reported for 4 patients (three grade 3 CRS and one grade 4 infusion-related reaction). Two events were considered as DLT: a grade 4 CRS which occurred after the second CYAD-02 infusion (i.e., first infusion of the consolidation cycle) and a grade 4 infusion-related reaction at DL1 which was retrospectively considered as DLT.

Clinical evaluation of CYAD-02 provides proof-of-concept of the miRNA-based construct against MICA/B to improve persistence of CAR T-cells in patients with AML/MDS

Importantly, when comparing the cellular kinetics of CYAD-02 in the CYCLE-1 trial with those of CYAD-01 in the DEPLETHINK trial at the dose-level 3 (Fig. 1B), we observed a prolonged persistence and increased engraftment with CYAD-02, suggesting that the shRNA targeting MICA/B effectively mitigates fratricide and enhances CAR T-cell persistence. Further analysis of individual kinetic parameters (Fig. 1C) revealed a significant difference between the two products in the time of last detectable measure of transgene copies/µg of DNA observed in peripheral blood post-infusion (T_{last}) with a mean of 34.50 days for DEPLETHINK versus 81.20 days for CYCLE-1 (p value of 0.0374), further supporting the superior persistence of CYAD-02. Comparable results were obtained when analyzing data from DL2, reinforcing these observations (Fig. S2).

CYAD-02 present a higher response rate and demonstrate better bone marrow blast reduction than CYAD-01 in patients with r/r AML/MDS

No objective responses were observed in the DEPLETHINK trial; however, two patients (28.5%) achieved stable disease (SD) (Fig. 2A and Table S2). Notably, one r/r AML patient who had SD also showed evidence of anti-leukemic activity, defined as a reduction of at least 50% in blast percentage following treatment with CYAD-01 (Fig. 2B).

Interestingly, treatment with CYAD-02 resulted in a 17% overall response rate (ORR), with two MDS patients at DL3 achieving a marrow complete remission (mCR) (Fig. 2A and Table S2). Additionally, SD was observed for eight patients (66%), half of whom showed evidence of anti-leukemic activity. Overall, 83.3% of treated patients experienced at least disease stabilization, and 50% demonstrated signs of anti-leukemic activity (Fig. 2A). Patients showed overall 21.65% lower blast percentage post CYAD-02 treatment than with CYAD-01 treatment after adjusting for baseline (95% CI: −42.55 to 0.7616)(Fig. 2B).

A dose-dependent response to CYAD-02 was also apparent, with DL3 yielding two objective responses (33% ORR) and three patients with SD (50%), including one patient with evidence of anti-leukemic activity (Fig. 2A and Table S2) – therefore selecting DL3 (i.e., 1×10^9 total cells per infusion) as the recommended dose. Despite these encouraging signs of anti-leukemic activity and disease control, most patients eventually relapsed within a few months.

Notably, the longest-lasting responses were observed in patients who either achieved a complete remission or who initially experienced disease stabilization and then received a second treatment cycle with CYAD-02, as outlines in the trial protocol. This second cycle, administered as three additional CYAD-02 infusions without preconditioning, appeared to reinforce the initial response to CYAD-02 and extend the period of disease control. Two patients underwent an allo-SCT following response to CYAD-02 treatment.

Strikingly, comparison of treatment responses between the CYCLE-1 and DEPLETHINK trials reveals a significant improvement in outcomes and blast reduction in patients treated with CYAD-02 versus those who received CYAD-01 (Fig. 2B). At dose levels 2 and

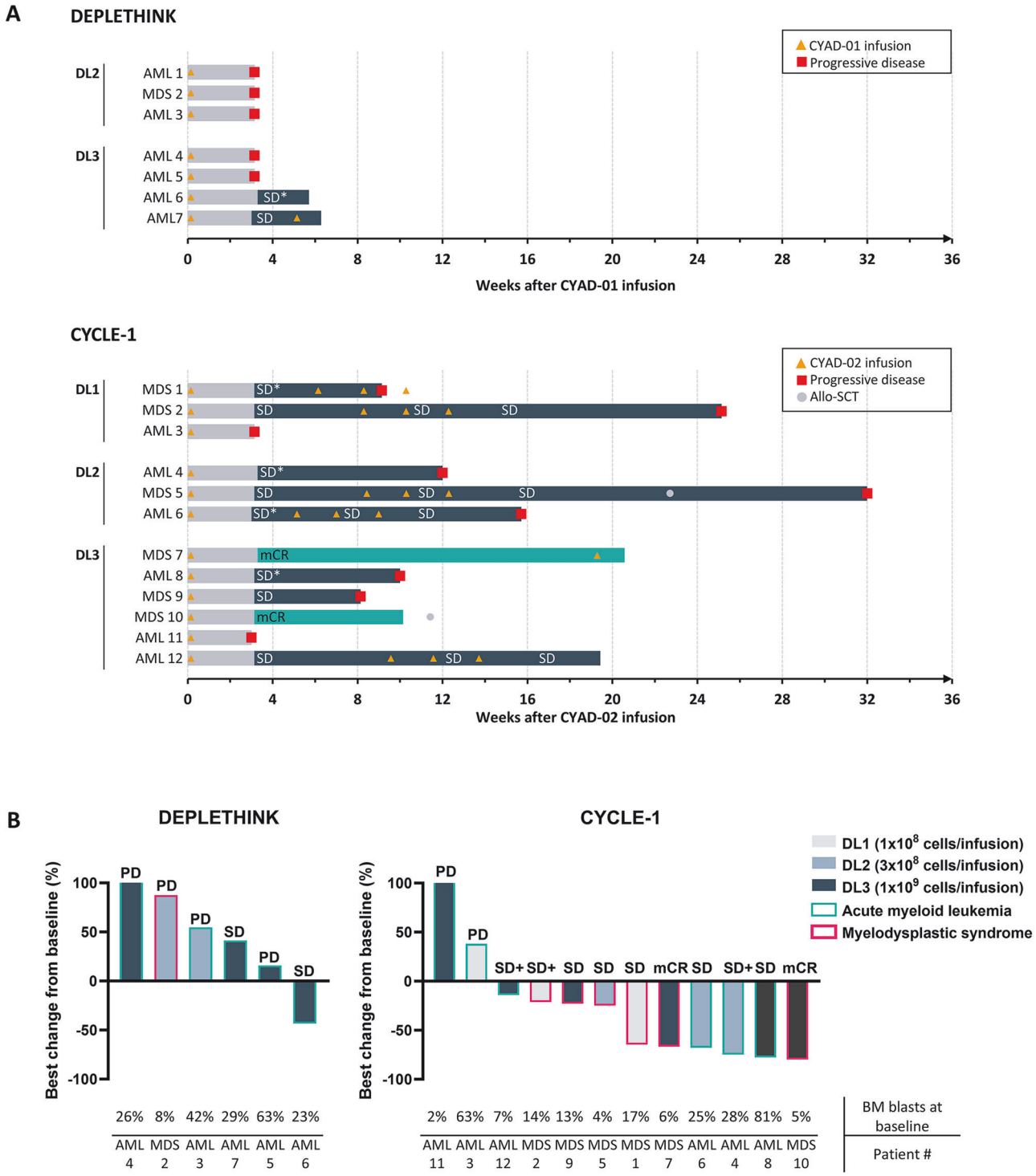

Fig. 2 Response to treatment in both DEPLETHINK and CYCLE-1 studies. A Time to Response and duration of responses. Each bar represents one patient. Data were censured at evidence of progression or patient withdrawal from trial. **B** Best change of bone marrow blasts from baseline. Absolute bone marrow bast percentages at baseline are indicated below the graph. ANCOVA analysis confirmed a significant treatment effect ($F(1,15) = 4.880$, $p = 0.0431$. All responses were confirmed and assessed according the Revised Recommendations of the International Working Group (IWG) for Diagnosis, Standardization of Response Criteria for AML patients [23], or IWG 2006 Uniform Response Criteria for patients with MDS [24]. Only patients who received the optimized manufacturing process are represented. Allo-SCT allogeneic hematopoietic stem cell transplantation, BM bone marrow, DL dose-level, mCR marrow complete remission, SD stable disease, SD*: SD with anti-leukemic activity i.e., more than 50% reduction in blast percentage post treatment.

3, the median overall survival was notably longer for CYAD-02 (21.71 weeks) compared to CYAD-01 (13.71 weeks, p value of 0.0473) (Fig. 3). Progression-free survival Kaplan Meier curves were also significatively different, with a median of 15.71 weeks for CYAD-02 in the CYCLE-1 trial versus 3.14 weeks for CYAD-01 in DEPLETHINK (p value of 0.0364) (Fig. 3).

Altogether, these findings suggest that the integration of the miRNA-based shRNA targeting MICA/B in CYAD-02 not only

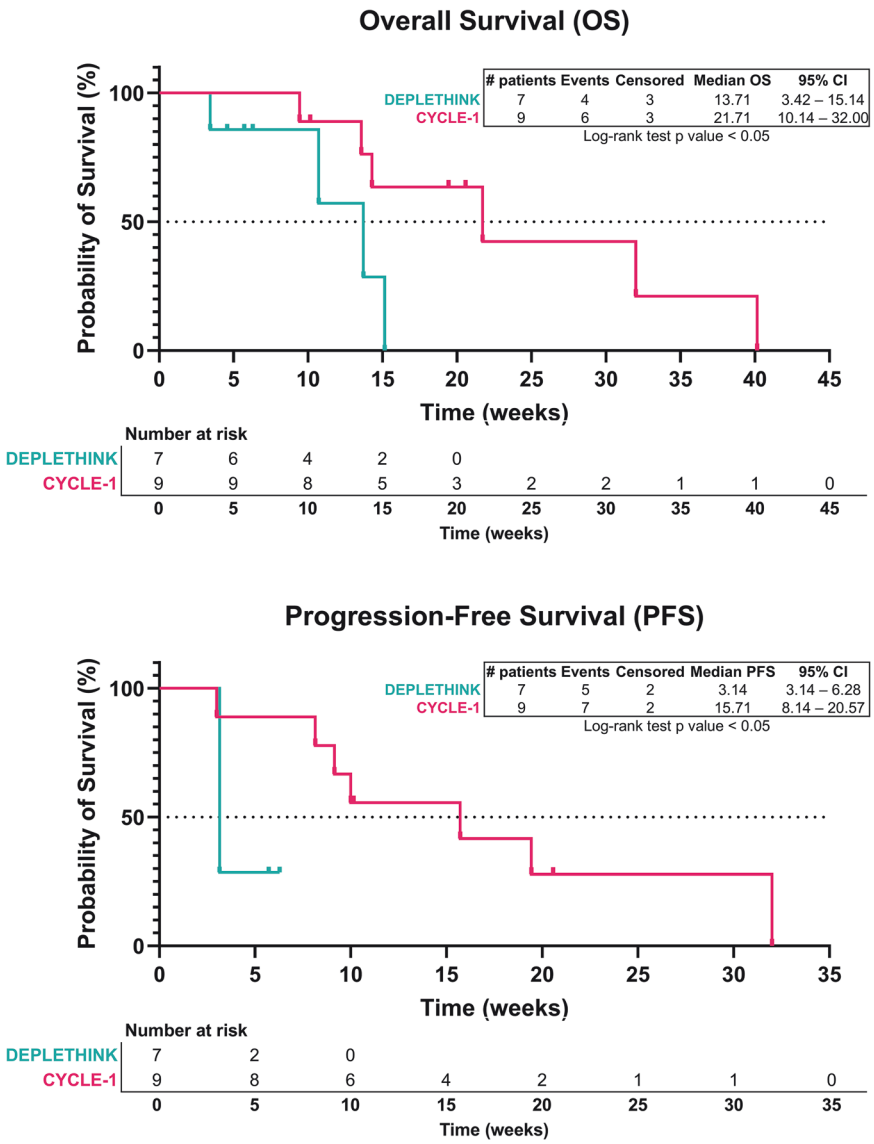

Fig. 3 Overall survival and progression-free survival in the DEPLETHINK and CYCLE-1 trials at dose-levels 2 and 3. Kaplan Meier overall survival (OS, upper panel; p value = 0.0473) and progression-free survival (PFS, lower panel; p value = 0.0364) for DEPLETHINK and CYCLE-1 trials. Only patients from dose-levels 2 and 3, and who received the optimized manufacturing process are represented. CI confidence interval.

enhances post-infusion persistence of the CAR T-cells but also significantly boosts their anti-tumor activity.

DISCUSSION

The therapeutic application of CAR T-cell therapy in AML and MDS remains limited due to the lack of leukemia-specific target antigens and the associated risk of on-target/off-tumor toxicity, antigen heterogeneity and a strong immunosuppressive TME [6, 27]. Current scFv-based CAR T-cell strategies have demonstrated limited clinical efficacy against AML and/or MDS adult patients, often accompanied by significant toxicity [8, 28]. For instance, CD123-directed CAR T-cells have shown modest response rates in adult patients with r/r AML (25% ORR) but high rates of CRS, including two grade 5 CRS [9]. CD33 and CLL1 CAR T-cells reached higher responses rates in young adults and pediatric patients with r/r AML (11–72% CR rates) but were also associated with high risk of grade ≥ 3 CRS and CRES [29, 30] and infections due to off-target toxicity [31]. Consequently, many clinical protocols include allo-SCT as a consolidation strategy post-CAR T-cell therapy to sustain remission but also rescue cytopenia [32, 33]. In this context, NKG2D-based CAR T-cells present a promising alternative due to their capacity to recognize eight distinct stress-induced ligands broadly expressed on malignant cells, but absent from healthy cells [34]. The multitargeted recognition may also reduce the risk of antigen escape and offer broader tumor coverage. Importantly, NKG2DL are also expressed on stromal cells such as endothelial cells from the tumor vasculature [32, 34] and on tumor-associated immune infiltrates like myeloid-derived suppressor cells and immunosuppressive regulatory T-cells from the TME [33, 35]—hence, supporting a multi-faceted mechanism of tumor clearance.

To address the limitations observed in early trials of NKG2D-based CARs [14], this study evaluated two strategies: (1) the use of lymphodepletion to improve CAR T-cell expansion and persistence (DEPLETHINK trial, CYAD-01), and (2) a novel miRNA-based shRNA strategy to silence MICA and MICB expression in the CAR T-cell product (CYCLE-1 trial, CYAD-02), aimed at mitigating fratricide.

Clinical data from CYCLE-1 trial indicate that CYAD-02 exhibited improved cellular kinetics compared to CYAD-01, supporting the hypothesis that autocrine ligand expression contributes to lack of

CYAD-01 persistence. Strikingly, the knockdown of MICA/B seemed also to contribute to an improved clinical activity of CYAD-02 as compared to the one achieved with CYAD-01. CYAD-02 indeed showed preliminary clinical activity with an ORR of 33% at the recommended dose (DL3) (2 patients with mCR out of 6) and 50% disease stabilization (3 patients out of 6)—encouraging findings in a high-risk AML/MDS population. Importantly, safety profile was favorable, with no neurotoxicity observed in the CYCLE-1 trial.

Despite these promising outcomes, limitations remain. While persistence of CYAD-02 was improved, overall expansion and duration of responses were modest, and somehow similar to those obtained with a multiple infusion schedule (3 biweekly infusions) with CYAD-01 [14]. A contributing factor may be the protocol re-dosing restriction that allowed a second cycle of treatment only in patients without detectable CYAD-02 in peripheral blood, potentially excluding individuals who would have benefited from further dosing. Future protocols may benefit from flexible re-dosing criteria based on clinical response rather than peripheral persistence. Additionally, these findings support the concept of using CAR T-cells as a bridge to allo-SCT transplant, a strategy increasingly adopted to maintain remission in AML patients post-CAR therapy [30, 36].

To further enhance CAR efficacy, additional engineering modules such as inducible cytokine secretion modulation or immune checkpoint inhibition or downregulation should be considered to help overcoming the immunosuppressive micro-environment in AML/MDS [19, –37, 38]. Similarly, modulating cytokines involved in AML progression or in CAR T-cell associated toxicities [9, 39], or multispecific approaches [40–42] may improve therapeutic performance.

Lastly, this study provides the first clinical proof-of-concept for the use of a single miRNA-based shRNA cassette to simultaneously knock down two independent genes, MICA and MICB, with functional benefit. This strategy could be broadly applicable across the CAR T-cell field, particularly in contexts where ligand expression contributes to fratricide or immune clearance [20]. Notably, MICA/B knockdown may also offer advantages during host immune system recovery following non-myeloablative preconditioning regimens, by reducing susceptibility to NK cell-mediated rejection—a consideration of relevance for other CAR T-cell therapies.

In summary, these findings support the feasibility and therapeutic potential of NKG2D-based CAR T-cells in AML, and highlight the importance of multimodal CAR T-cell engineering to overcome disease- and product-specific limitations, providing a platform for future optimization and clinical development.

DATA AVAILABILITY

Upon email request (contactus@celyad.com), individual participant de-identified datasets will be made available on a case-by-case basis. Supporting documents that will be available upon request include the study protocol(s), the informed consent form(s), and statistical analysis plan(s).

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ACKNOWLEDGEMENTS

We thank all former colleagues at Celyad Oncology who might have contributed to this work. The two clinical trials (CYCLE-1 and DEPLETHINK) were sponsored by Celyad Oncology SA, which provided all the trial materials. We thank the patients who participated in the trials, their families, friends, and caregivers, but also the trial staffs and healthcare providers at all the clinical trial sites for their commitment and support during the trial conduct.

AUTHOR CONTRIBUTIONS

DP, TK, DD, YB, TLL, DAS, SA, and WGB were responsible for the screening, enrollment and treatment of patients, and gathered data. CL and AF were responsible for designing the clinical protocol, extracting and analyzing data, and interpreting results. CL wrote the original draft manuscript, AF and EB reviewed data compiled in the draft manuscript and provided scientific input. All authors participated in the writing of the manuscript, provided feedback, and approved the final submitted version.

COMPETING INTERESTS

DP, TK, DD, YB, TLL, DAS, SA, and WGB were Investigators of the DEPLETHINK or CYCLE-1 clinical trials, sponsored by Celyad Oncology SA. DD: Consultancy for Novartis, BMS, Incyte, Alexion, Sobi, Takeda, Roche, Servier, CSL Behring; research funding by Alexion, Sobi, Roche, Novartis, Amgen. TLL: Consulting or advisory role: Servier, Jazz Pharmaceuticals, Syndax, Daiichi Sankyo; research funding to the institution: Bio-Path Holdings, Inc, Astellas Pharma, Celyad Oncology, Aptevo Therapeutics, Cleave Biosciences, CicloMed, Jazz Pharmaceuticals, Cardiff Oncology, Kura Oncology. DAS: Research funding from Aprea; advisory board member at AbbVie, Agios, Aprea, Bristol Myers Squibb, Intellia, Kite, Novartis, Shattuck Labs, and Syndax; consultant to Magenta, Novartis, and Takeda; speakers' bureau at Bristol Myers Squibb and Incyte. AF, EB, and CL were employees of Celyad Oncology SA during the realization of this work. The research conducted within the manuscript may lead to the development of products which may be licensed by Celyad Oncology SA. The other authors declare no conflicts of interest.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41375-025-02767-4.

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