NKG2D-based chimeric antigen receptor therapy induced remission in a relapsed/refractory acute myeloid leukemia patient

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doi:10.3324/haematol.2017.186742

Supplementary information for:

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Supplementary Figures

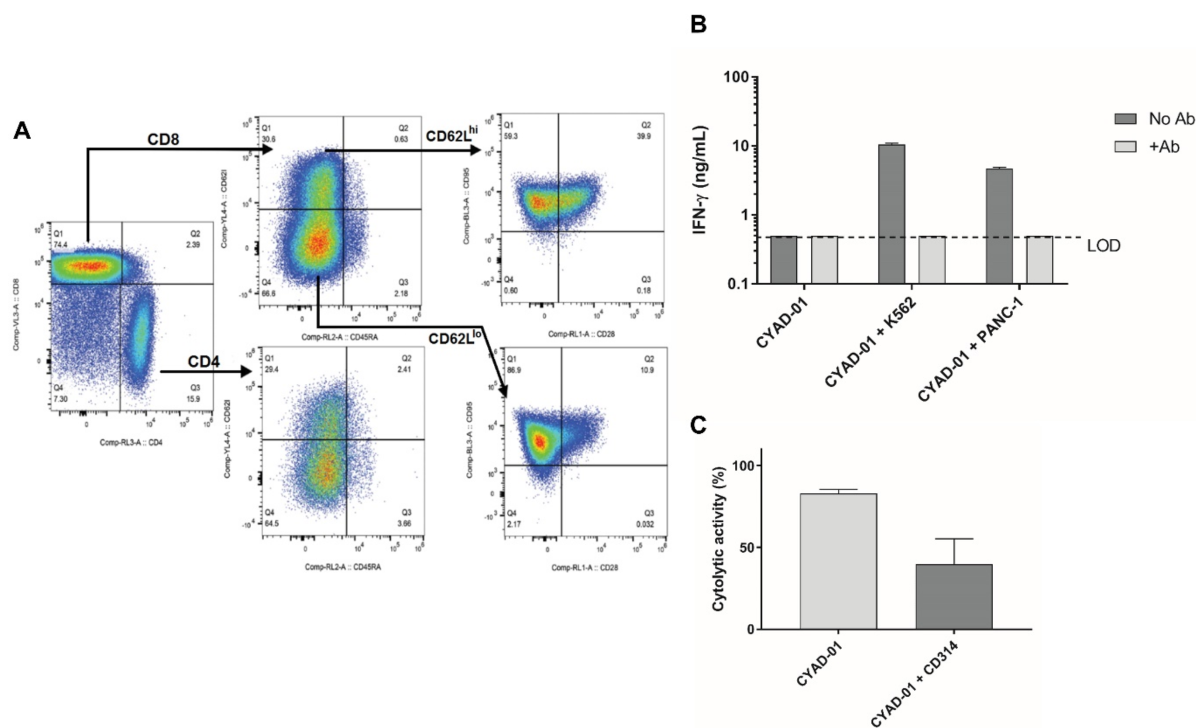

Fig. S1: Patient's CYAD-01 phenotype and *in vitro* functionality. (A) A sample of the infusion product was analyzed for cell subset composition and memory phenotype. $CD3^{+}$ viable singlet cells showed a high CD8 preponderance with both CD4 and CD8 subsets possessing a majority of $CD62L^{lo}$ phenotype. 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-γ 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, *: $p < 0.05$.

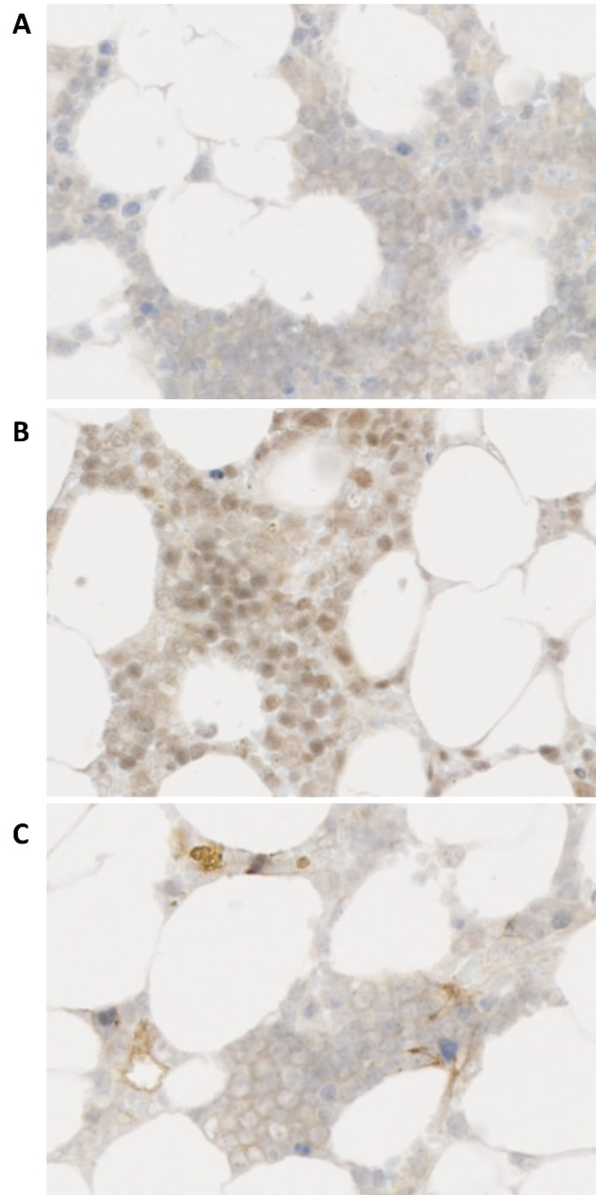

Fig. S2: Immunohistochemistry analysis of NKG2D ligand expression in bone marrow sample taken before first CYAD-01 injection. One BM biopsy was subject to a range of IHC staining protocols specific for the following NKG2DL: MICA/MICB, ULBP1, ULBP2/5/6, ULBP3. In addition, a sample of each was prepared 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 (**TH**erapeutic **I**mmunotherapy with **NK**R-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 (exclusing 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.