Time: 2024.05.26-2024.05.31

1. **Experiment:** LDH assay for measuring cytotoxic activity
2. **Time:** 2024.05.26-2024.05.31
3. **Member:** Xinyu Zhu, Yaqi Gao, Xiaoyuan Chen
4. **Materials:** Raji cell line, HepG2 cell line, AsPC-1 cell line, LDH assay kit.
5. **Method:**

(1) Cell Preparation:

①Seed cells into culture dishes with the appropriate medium. Incubate at 37°C with 5% CO_2 for 2 days. Change to a fresh medium the evening before the experiment.

②Adjust the density of effector cells to 1.0×10^4 cells/mL and tumor cells to 2.0×10^3 cells/mL.

(2) Cytotoxicity Assay:

①Prepare a 96-well round-bottom plate for the cytotoxicity assay. Conduct experiments at E:T ratios of 5:1 and 2:1, with replicates per ratio. Add effector and target cells to each well, with a total volume of 100 μL per well.

②Set up control wells: Natural release control wells with the same number of effector cells as in the experimental wells. Maximum release control wells with the same number of target cells, adding medium to achieve a final volume of 90 μL per well. Natural release control wells for target cells, adding medium to achieve a final volume of 100 μL per well. Background control wells with 100 μL of CAR-NK cells.

③Add 10 μL of sterile ultrapure water to the spontaneous release control wells for both effector and target cells.

④Incubate the plate at 37°C with 5% CO_2 for 4 hours.

⑤Add 10 μL of lysis buffer to the maximum release control wells and incubate for 45 minutes.

⑥Centrifuge at 250×g for 3 minutes. Transfer 50 μL of supernatant from each well to a corresponding flat-bottom plate. Add 50 μL of reaction substrate to each well and incubate in the dark at room temperature (25°C) for 30 minutes. Once the color deepens, add 50 μL of stop solution to each well and gently mix the plate.

⑦Using a microplate reader and measure the absorbance at 490 nm and 680 nm. Subtract the absorbance value at 680 nm (background signal) from the absorbance at 490 nm (D).

⑧For accurate calculations, subtract the background control average value from the experimental average, the effector cell spontaneous release control value, and the target cell spontaneous release control value.

⑨Calculate the NK cell killing rate (%) using the following formula:

NK killing rate (%)=Target cell lysis OD value−Target cell spontaneous release OD valueExperimental OD value−Effector cell spontaneous release OD value−Target cell spontaneous release OD value−Back ground control average value×100%

6. **Result:**

Co-culturing with CD19-positive Raji cells without exogenous IL-2, the percent of LDH release of NK92 cells transduced with αCD19 eCAR sequence was significantly higher than NK92 cells without transduction or transduced with CAR sequence, which was observed at effector-to-target (E:T) ratio of both 5:1($P < 0.001$) and 2:1 ($P < 0.01$). Similar results were observed when NK92 cells transduced with αCD20 eCAR sequence were co-cultured with CD20-positive Raji cells ($P < 0.001$ for E:T ratio of 5:1 and < 0.05 of 2:1). Interestingly, in co-culture with GPC3 positive HepG2 cells, a significantly higher percent of LDH release in NK92 cells transduced with αGPC3 eCAR sequence was observed in both the presence ($P < 0.01$ for E:T ratio for 5:1 and < 0.05 for 2:1) and absence of IL-2 ($P < 0.001$ for E:T ratio of 5:1 and of 2:1). Similar to results in co-culture with CD19-positive Raji cells, elevated LDH release percent was observed in NK-92 cells transduced with αMSLN eCAR without exogenous IL-2 supplement ($P < 0.05$ for E:T ratio of 5:1 and < 0.001 of 2:1).

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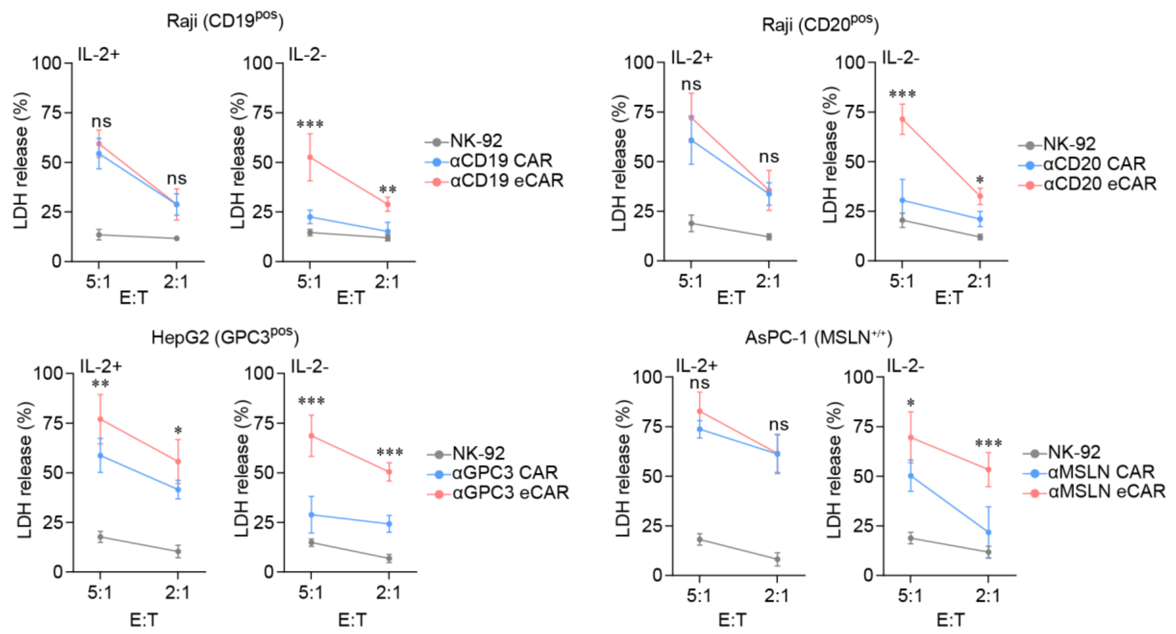

Fig.1 Lysis of different tumor cells at various effector-to-target ratios