Time: 2025.09.10-2025.09.15

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1. **Experiment:** Flow cytometric analysis of NK cell PFN & GrB under acidic condition
2. **Time:** 2025.09.10-2025.09.15
3. **Member:** Xudong Tang, Yang Jin, Binxuan Zhang, Kaiqing Zhang, Xuantong Liu
4. **Materials:** Flow cytometer, Anti-human Perforin antibody (BioLegend 308104), anti-Human Granzyme B Antibody (BioLegend 515404), Ice-cold PBS Staining buffer, Brefeldin A, 4% paraformaldehyde (PFA), 0.1% Triton X-100
5. **Method:**

(1) Cell preparation:

① Co-culture target cells (K562) in logarithmic growth phase with effector cells (pre-stimulated NK cells) at a specific effector-to-target ratio in a 37 ℃, 5% CO_2 incubator, under acidic condition of 15 mM lactic acid (pH 6.4), and neutral pH of 7.4 respectively.

② Add Brefeldin A (diluted 1:1000) to the co-culture system 12 hours after initiation. Continue culture for a total of 46 hours.

③ After co-culture, collect cells into flow cytometry tubes. Wash once with pre-chilled PBS, centrifuge at 300 × g for 5 minutes, and discard the supernatant.

④ Resuspend cells in 100 μL flow cytometry staining buffer for cell counting.

(2) Surface Staining

① Dosage example for a 100 μL reaction system: CD3-PerCPCy5.5 (1:100), CD56-PE (1: 100).

② Add antibodies to the cell tube, gently mix by pipetting, and incubate at 4 ℃ in the dark for 30 minutes.

③ Wash with 2 mL staining buffer, centrifuge at 300 × g for 5 minutes, and discard supernatant. Repeat washing once.

(3) Fixation and Membrane Permeabilization

① Resuspend cells in 100 μL fixative, incubate at room temperature in the dark for 15-20 minutes.

② Add 2 mL staining buffer, centrifuge, discard supernatant, and thoroughly remove fixative.

③ Resuspend cells in 100 μL lysis buffer, incubate at room temperature in the dark for 15-20 minutes.

(4) Intracellular Staining

① Dilute intracellular antibodies in lysis buffer: Perforin-FITC (1:20), Granzyme-BV421 (1: 20). Centrifuge and discard lysis buffer.

② Add the intracellular antibody cocktail to the cell tube, gently pipette to mix, and incubate at room temperature in the dark for 30-45 minutes.

③ Wash with 2 mL lysis buffer, centrifuge at 300 × g for 5 minutes, and discard the supernatant. Repeat the wash once.

④ Resuspend cells in 300-500 μL flow cytometry staining buffer and analyze immediately.

(5) Data Analysis:

① The suspension was transferred to flow cytometry sample tubes.

② Analysis strategy:

Gate to exclude doublets (using FSC-A vs FSC-H).

Gate to exclude dead cells (using a viability dye).

Identify the target cell population (NK cells: $CD3^-$ $CD56^+$).

Analyze PFN and GrB expression: percentage of positive cells (%) and MFI.

6. Result:

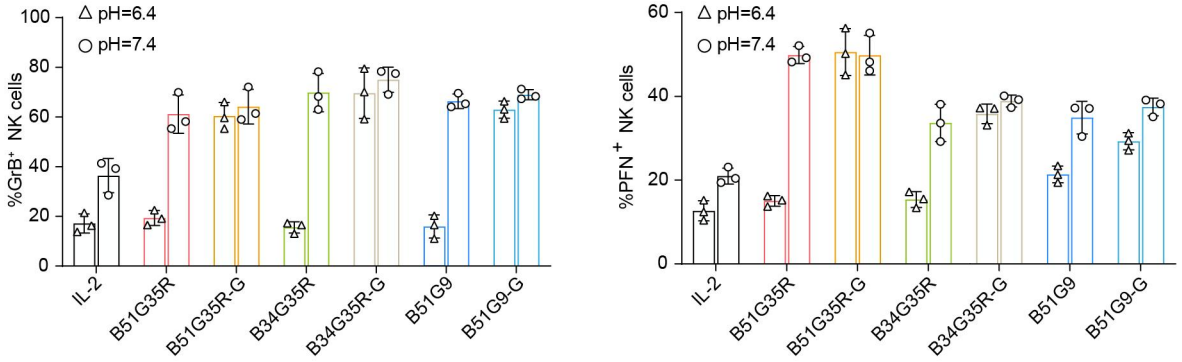

Figure.1 PFN, GrB detection of NK cells pre-activated by IL-2, B51G35R, B51G35R-G, B34G35R, B34G35R-G, B51G9, B51G9-G separately at both pH=6.4 and pH=7.4. Data are representative of at least three independent experiments (**$P < 0.01$, *$P < 0.05$).