A Robust, Xeno- and Feeder-Free Culture System for Expansion of Human Peripheral Blood NK Cells

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INTRODUCTION

Natural killer (NK) cells are lymphocytes that play an important role in innate immunity against tumors and viral infections by secreting proinflammatory cytokines and exhibiting cytotoxicity. Clinical trials have shown that NK cells are able to exert clinical activity against tumors without causing side effects as observed with T-cell therapy including graft-vs-host-disease (GvHD), neurotoxicity or cytokine release syndrome. Therefore, NK cells are an attractive tool for the development of cancer immunotherapies against various tumors. NK cells are present in peripheral blood (PB) but their numbers are not sufficient for therapeutic applications. We have developed a culture system for expansion of PB NK cells without feeder cells, serum, or xeno components. Freshly isolated NK cells were cultured for two weeks in ImmunoCult™ NK Cell Expansion Medium and on plates coated with ImmunoCult™ NK Cell Expansion Coating Material. $CD56^+CD3^-$ NK cells expanded 89 ± 17-fold (n = 34, mean ± SEM), with an average frequency of 87 ± 1%. The expanded NK cells were functional; they degranulated and secreted both interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) when stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin, or co-cultured with K562 cells. NK cells were able to kill target K562 cells in direct killing. They also killed SK-BR-3 adenocarcinoma cells in an antibody-dependent cellular cytotoxicity (ADCC) manner. Therefore, this culture system enables xeno- and feeder-free generation of large numbers of functional NK cells for basic and clinical research.

METHODS

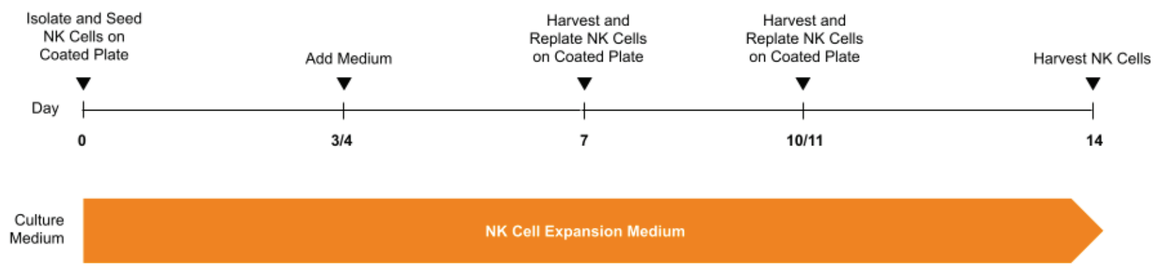

FIGURE 1. Culture Protocol

Protocol for Expansion of NK Cells
NK cells were isolated from human peripheral blood (PB) leukapheresis samples using EasySep™ Human NK Cell Isolation Kit. Freshly isolated $CD56^+$ NK cells were seeded at 1 x 10^6 cells/mL in NK Cell Expansion Medium (ImmunoCult™ NK Cell Base Medium supplemented with ImmunoCult™ NK Cell Expansion Supplement) into 24-well plates (0.5 mL/well) coated with ImmunoCult™ NK Cell Expansion Coating Material. On day 3 or 4, an equal volume of fresh medium was added. On day 7, and again on day 10 or 11, expanding NK cells were harvested, diluted, and seeded at 2 x 10^5 cells/mL in NK Cell Expansion Medium on freshly coated plates. Finally, on day 14 the expanded NK cells were harvested and characterized for phenotype or functionality in appropriate downstream assays.

Phenotyping of NK Cells
Cultured cells were harvested, counted, and analyzed by flow cytometry for expression of CD56, NKp46, NKp44, NKp30, NKG2D, KIR, CD3, CD94, and CD16. Staining for killer cell immunoglobulin-like receptor (KIR) molecules was performed using two different antibody clones, HP-MA4 and 180704, which recognize distinct KIR molecules. Dead cells were excluded by light-scatter profile and DRAQ7™ staining. The yield of culture-expanded NK cells ($CD56^+CD3^-$) was calculated from the fraction of cells expressing the specified markers.

Degranulation and Cytokine Production Assay
Expanded NK cells were left unstimulated (control) or were stimulated with either phorbol 12-myristate 13-acetate (PMA) and ionomycin or target cells at a ratio of 1:1 effector:target cells. CD107a antibody was added, and cultures were incubated at 37°C for 4 hours. After the first hour, Monensin and Brefeldin A were added. Cells were assessed for surface CD56, CD107a, and intracellular IFN-γ and TNF-α expression by flow cytometry.

Image-Based Killing Assay
Expanded NK cells were co-cultured with Incucyte® Cytolight Rapid Dye-labeled target cells at 1:1 ratio of NK:target cells at 37°C for 4 hours. Incucyte® Caspase-3/7 Dye, a caspase-inducible dye, was added to the co-culture to detect caspase-induced apoptosis of the target cells. Images were obtained every hour using the Incucyte® imaging system and then analyzed to determine % killing ([# apoptotic target cells ÷ # total labeled target cells] * 100).

RESULTS

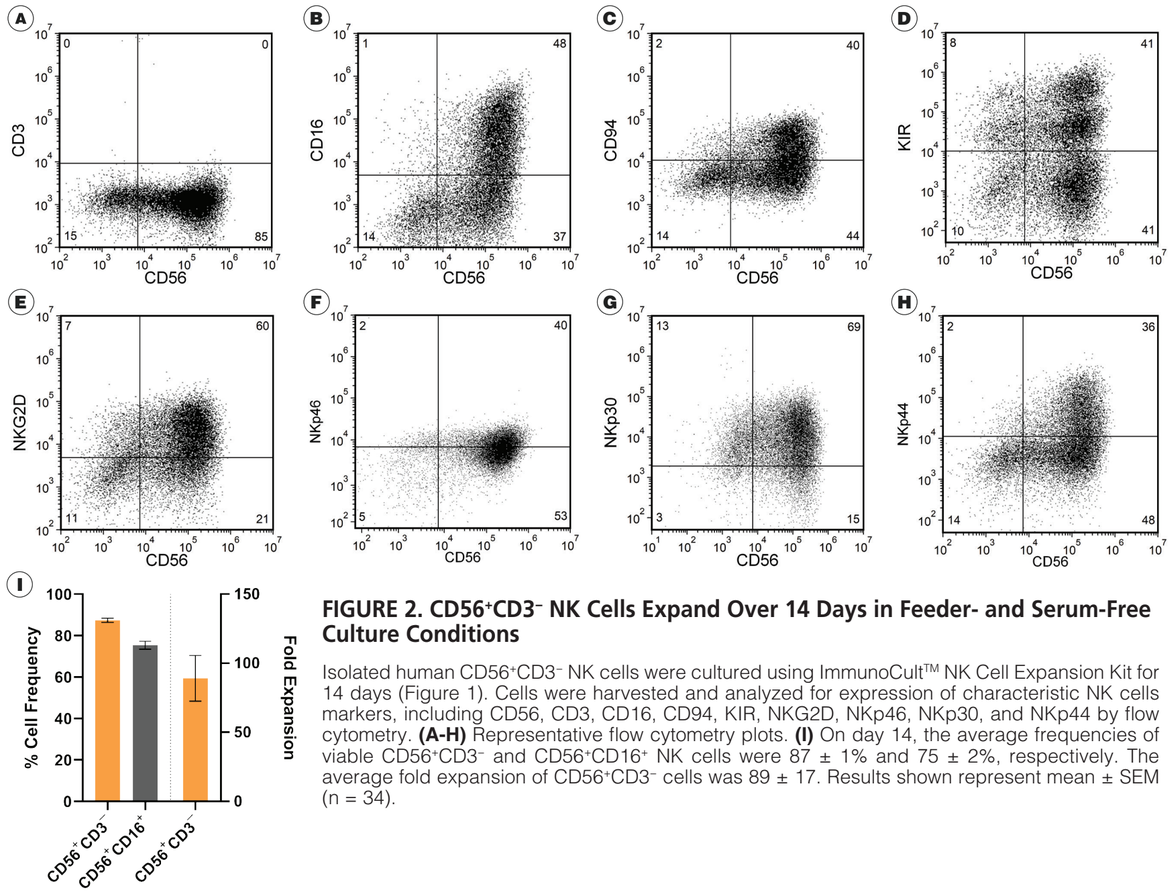

FIGURE 2. $CD56^+CD3^-$ NK Cells Expand Over 14 Days in Feeder- and Serum-Free Culture Conditions

Isolated human $CD56^+CD3^-$ NK cells were cultured using ImmunoCult™ NK Cell Expansion Kit for 14 days (Figure 1). Cells were harvested and analyzed for expression of characteristic NK cells markers, including CD56, CD3, CD16, CD94, KIR, NKG2D, NKp46, NKp30, and NKp44 by flow cytometry. **(A-H)** Representative flow cytometry plots. **(I)** On day 14, the average frequencies of viable $CD56^+CD3^-$ and $CD56^+CD16^+$ NK cells were 87 ± 1% and 75 ± 2%, respectively. The average fold expansion of $CD56^+CD3^-$ cells was 89 ± 17. Results shown represent mean ± SEM (n = 34).

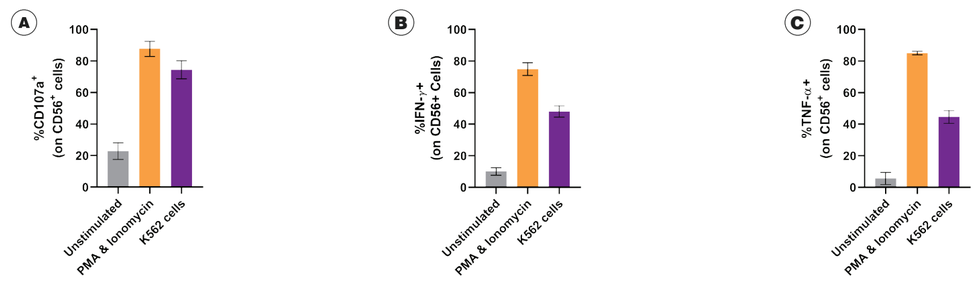

FIGURE 3. Expanded NK Cells Can Degranulate, and Produce Cytokines After Stimulation

Isolated $CD56^+CD3^-$ NK cells were expanded for 14 days (Figure 1) and then assessed for degranulation and cytokine production. **(A)** The average frequency of NK cells expressing surface CD107a, a marker of degranulation, was 23 ± 5% for the unstimulated control, 88 ± 5% after stimulation with PMA and ionomycin, and 74 ± 6% after stimulation with K562 cells. **(B)** The average frequency of NK cells expressing intracellular IFN-γ was 10 ± 2% for the unstimulated control, 75 ± 4% for cells stimulated with PMA and ionomycin, and 48 ± 4% for cells co-cultured with K562 cells. **(C)** The average frequency of NK cells expressing intracellular TNF-α was 6 ± 4% for the unstimulated control, 85 ± 1% cells stimulated with PMA and ionomycin, and 45 ± 4% for cells co-cultured with K562 cells. Data represent mean ± SEM (n = 6 - 13).

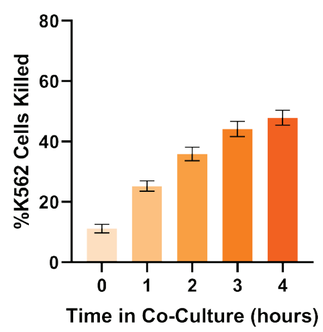

FIGURE 4. Expanded NK Cells are Functional, Killing K562 Cells in Co-Culture

Isolated $CD56^+CD3^-$ NK cells were expanded and their cytotoxicity against K562 target cells was assessed using an image-based killing assay. In this assay an average of 48 ± 2% K562 target cells were killed after 4 hours of co-culture with expanded NK cells. Data represent mean ± SEM (n = 9).

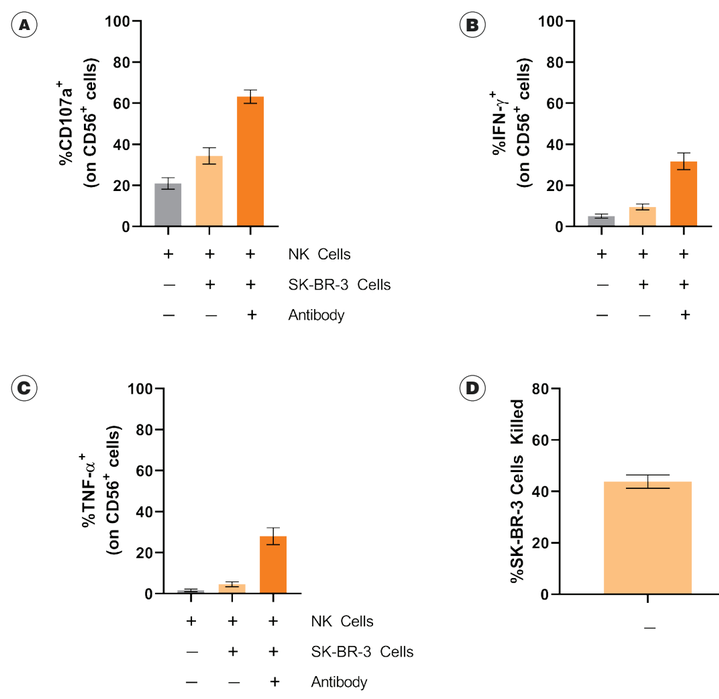

FIGURE 5. Antibody-Dependent Stimulation Induces Degranulation, Cytokine Production and Enhances Cytotoxic Activity

Isolated $CD56^+CD3^-$ NK cells were expanded for 14 days (Figure 1). Expanded NK cells were left unstimulated, or co-cultured at a ratio of 1:1 effector:target cells, with either untreated SK-BR-3 adenocarcinoma cells or SK-BR-3 cells that were previously incubated with a human anti-HER2 antibody at 37°C for 30 minutes. Both a degranulation and cytokine production assay **(A-C)** and an image-based killing assay were performed **(D)**. **(A)** The average frequency of NK cells expressing surface CD107a was 21 ± 3% for the unstimulated control, 34 ± 4% after co-culture with SK-BR-3 cells, and 63 ± 3% after co-culture with SK-BR-3 cells preincubated with antibody. **(B)** The average frequency of NK cells expressing intracellular IFN-γ was 5 ± 1% for the unstimulated control, 10 ± 1% for cells co-cultured with SK-BR-3 cells, and 32 ± 4% for cells co-cultured with SK-BR-3 cells preincubated with antibody. **(C)** The average frequency of NK cells expressing intracellular TNF-α was 2 ± 1% for the unstimulated control, 5 ± 1% for cells co-cultured with SK-BR-3 cells, and 28 ± 4% for cells co-cultured with SK-BR-3 cells preincubated with antibody. **(D)** After 4 hours, an average of 44 ± 3% SK-BR-3 cells were killed, while ADCC in cells pretreated with antibody increased this to 60 ± 2% killed. Data represent mean ± SEM (n = 7).

Summary

- Human PB-derived NK cells yield on average 89-fold expansion of $CD56^+CD3^-$ cells in serum- and feeder-free conditions after 14 days
- Expanded $CD56^+$ NK cells express NK cell markers CD56, CD16, NKG2D, NKp46, NKp30, NKp44, CD94, and KIR
- Expanded NK cells are functional, as follows:
  - They degranulate and produce IFN-γ after stimulation with either target K562 cells or PMA & ionomycin
  - They kill K562 cells in co-culture as visualized using caspase-3/7 activity
  - They are capable of responding to target cells in an antibody-dependent manner
- This culture system enables research into the development of cellular immunotherapy.