Efficient feeder cell-free *in vitro* expansion of NK cells for CAR-NK therapies using human platelet lysate

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Introduction

- Building on the successes and addressing the limitations of CAR-T cell therapy, researchers are now exploring the use of natural killer (NK) cells as a potentially stronger candidate for the CAR based platform. While T cells are part of the adaptive immune system, and need to be exposed and selectively activated and enriched after contact with exogenous or abnormal antigens in the body, NK cells are part of the innate immune system and exhibit inherent anti-tumor activity without requiring prior sensitization, making them attractive targets for CAR engineering (CAR-NK cells). By combining the potent cytotoxicity of NK cells with the specificity of CARs, CAR-NK therapies aim to provide a safer and potentially more broadly applicable form of cellular immunotherapy. In the last 5 years, there have been over 50 worldwide clinical trials registered in ClinicalTrials.gov using CAR-NK cells. The data shows that the use of CAR-NK cells leads to a lower incidence and severity of the cytokine release syndrome (CRS). Additionally, NK cells offer less obstacles than T cells for the development of allogeneic "off the shelf" approaches, which would result in a more consistent and reliable therapy.

- Despite CAR-NK's higher safety and efficiency profile, the difficulty of obtaining clinically relevant numbers of CAR-NKs limits their clinical use. NK cells are much less abundant that T cells, constituting just 15% of the total peripheral blood lymphocytes on average. Additionally, NK cells have a much shorter life span and lower expansion capabilities *in vitro*. Currently, the most efficient method to expand NK cells depends on the co-culture with a feeder cell line for the activation and proliferation of NK cells in order to produce significant yields of CAR-NKs . Even when feeder cells (normally a tumor cell line) are used to promote NK proliferation, the efficiency and success rates are often low and variable patient to patient. Besides the low efficiency rates, the use of tumor cell lines for NK production constitutes a technical and regulatory challenge.

- We developed a protocol that involves the use of human platelet lysate (hPL) for *in vitro* maintenance, manipulation and expansion of human NK cells that, without the need of co-culturing with feeder cells of any type, enables robust *in vitro* expansion of human NK cells, surpassing the yields achieved with current state-of-the-art methodologies. NK cells expanded *in vitro* using our hPLs PLTMax® (first generation hPL that requires the use of heparin to avoid clot formation) and PLTGold® (second generation hPL completely xeno-free and clot free) showed better phenotypic and functional characteristics than cells expanded using AB Serum and feeder cells, as demonstrated through flow cytometry and tumor cytotoxicity assays, respectively.

Results I

Ia. Isolation, expansion and characterization of NKs using PLTMax® and PLTGold®

NKs from healthy donors were isolated using an immunodensity negative selection cocktail (RossetteSep ™ Human NK Cell Enrichment Cocktail, STEMCELL™ Technologies) (Figure 1A) and expanded using RPMI supplemented at 5% with PLTMax®, PLTGold® or human AB serum plus K562 feeder cells. IL-2 and IL-15 (Sigma-Aldrich) were added for all conditions at 100U/mL and 10ng/mL respectively. As seen in Figure 1B, both PLTMax® and PLTGold® produced higher NK cell yields after 2 weeks of culture than the classic supplementation with 5% human AB Serum and feeder cells.

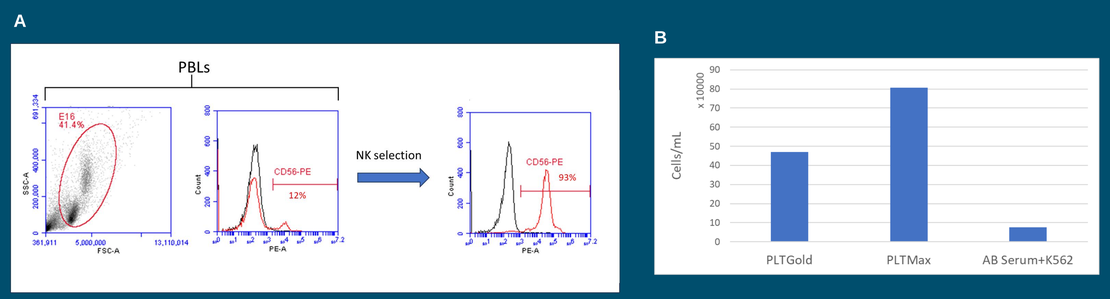


Figure 1. Isolation and expansion of donor NKs using hPL. A) NK cell population as determined by CD56 expression in peripheral blood leukocytes (PBLs) from healthy donor before and after enrichment of NK cell population. B) Cells numbers obtained after a week of culture for NKs expanded in RPMI medium supplemented with, IL-2, IL-15 and either PLTMax®, PLTGold® or human AB Serum+K562 feeder cells.

Besides higher cell yields, NK cells expanded *in vitro* using our hPLs showed higher expression of CD56 and CD16 than NK cells expanded using feeder cells, as demonstrated through flow cytometry (Figure 2).

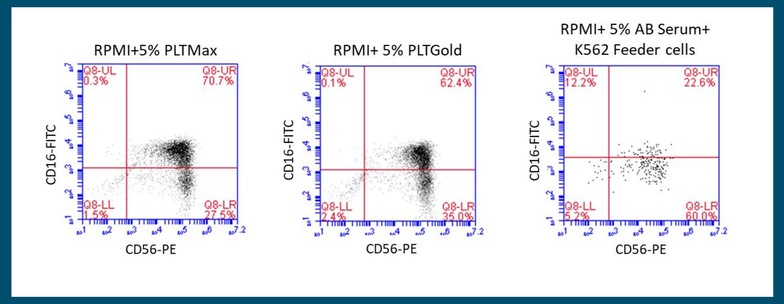


Figure 2. Phenotypic characterization of NK cells expanded using PLTGold® and PLTMax® Analysis by flow cytometry of CD56+ and CD16+ populations. Histograms show the distribution of the expression and co-expression of CD56 and CD16 in cultures of NK cells selected from human peripheral blood.

Results II

Ib. Isolation, expansion and characterization of NKs using PLTMax® and PLTGold®

Even in cases where donors had low NK count and the NK purification was suboptimal (Figure 3A), expansion of selected NK cells for a week using human platelet lysate resulted in pure NK populations where almost 100% of the cells were CD3-CD56+ (Figure 3B). CD16 levels also increased for NKs of all donors evaluated after expansion with PLTGold® (Figure 3B).

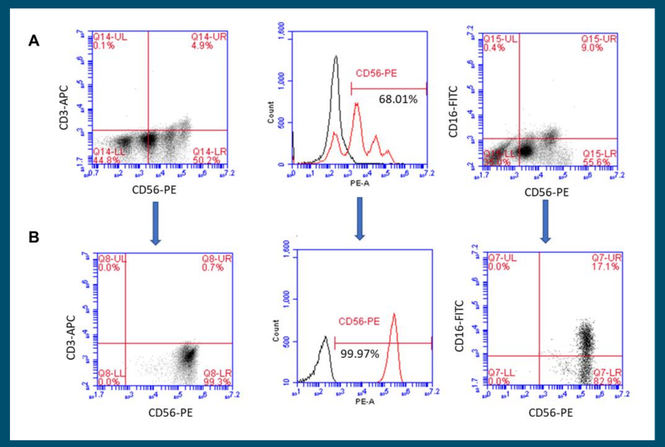
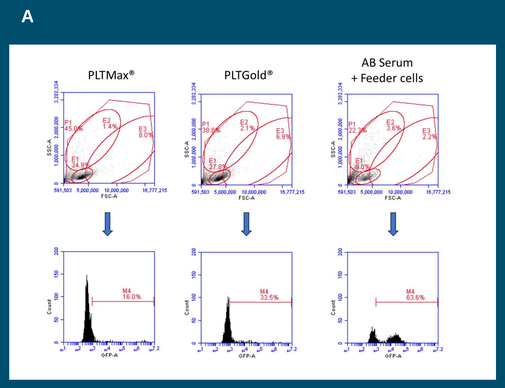


Figure 3. Characterization of NKs expanded with PLTGold®. A) Phenotype of NK cells isolated from a young donor with a suboptimal yield of 68% CD56+ cells. B) Phenotype of NK cells isolated with a suboptimal yield after expansion for a week using RPMI supplemented with 5% PLTGold®, IL-2 and IL-15.

II. In vitro NK cell tumor cytotoxicity assay

Tumor cytotoxicity assessment was performed by co-culturing K562 cells expressing green fluorescence protein (GFP) with NKs expanded for 1 week using RPMI supplemented with PLTGold®, PLTMax® or AB serum plus K562 cells. Cell mixtures were incubated overnight before assessing GFP levels through flow cytometry to determine viability of tumor cells. As seen in figure 4A, the strongest tumor cytotoxicity was obtained when co-culture of tumor cells was done with NKs previously expanded in PLTMax®, followed by NK cells expanded with PLTGold® as indicated by the lower levels of GFP found in the co-cultures in comparison to co-cultures with NK cells expanded using human AB serum and feeder cells. Real time cell imaging was performed over the course of 24h to monitor the cytotoxic effects of NK cells previously expanded in media supplemented with either PLTGold® (Figure 4B), PLTMax® or human AB serum and K562 cells.



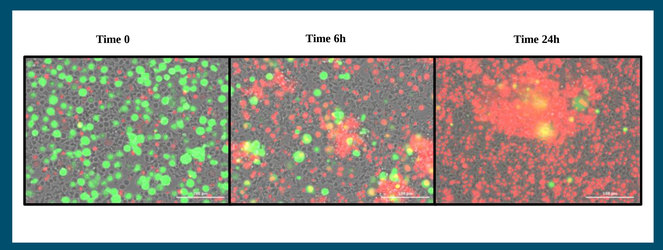


Figure 4. Cytotoxicity assay using NKs expanded in media supplemented with PLTGold® and PLTMax®. A) Flow cytometry histograms showing GFP expression in overnight co-cultures of K562-GFP and NK cells expanded with human platelet lysates *vs* human AB serum and K562-GFP feeder cells. B) Fluorescence imaging for co-cultures of K562-GFP cells and NK cells previously expanded in RPMI supplemented with PLTGold® and stained with eLive Red.

Conclusions

- NK cells expanded using media supplemented with human platelet lysates PLTMax® and PLTGold® exhibited higher proliferative capacity than NK cells expanded using the traditional approach supplementing with human AB Serum and feeder cells.
- Besides higher cell yields, NK cells expanded using human platelet lysate showed a better phenotype, with higher levels of CD56+ CD16+ cells.
- Expansion of NK cells using human platelet lysate resulted in higher NK cell yields even for donors that had lower numbers of NK populations and suboptimal NK cell isolations, therefore facilitating potential treatment of all patients in autologous CAR-NK therapies regardless of initial cell yields.
- NK cells expanded using media supplemented with human platelet lysates PLTMax® and PLTGold® exhibited higher cytotoxicity effects than those expanded using human AB Serum and feeder cells.
- PLTMax® demonstrated overall to be the best option to obtain the highest cell yields, highest number of CD56+ and CD16+ cells and therefore highest cytotoxic effects in vitro. However, PLTGold® offers the advantage of a fully xenogeneic free media supplement since it doesn't require the addition of heparin to the cell culture media.

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