# Gene expression analysis reveals mechanisms with potential relevance to NK cell expansion protocols for immunotherapy

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## Introduction

Natural killer (NK) cells hold great potential for adoptive anti-cancer immunotherapies. However, for therapeutic approaches, large numbers of functional NK cells are required, and therefore effective protocols to expand these cells *ex vivo* need to be established. Current expansion protocols lead to variable efficiencies in the generation of NK cells, and it is not completely understood which cell culture parameters result in good or poor proliferative conditions. Therefore, we decided to investigate the molecular mechanisms regulating NK cell proliferation by gene expression and functional annotation analysis of *ex vivo* expanded NK cells.

We used a co-culture system with autologous irradiated feeder cells, which provides a microenvironment of multiple signals that potentially influence NK cell proliferation. In the co-culture, NK cell expansion was higher after 5–7 days compared to a standard cultivation method using only IL-2 supplement. This led us to define day 5 as the most suitable time point to study early events driving NK cell proliferation. Using whole genome microarray gene expression analysis we compared NK cells showing high and low proliferative activity and non-cultured control samples from five different healthy donors.

## Methods

Purified NK cells were labeled with a cell trace dye and then expanded in a co-culture with autologous irradiated, activated PBMCs (IAP). Figure 1 shows an overview of the protocol. On day 5, NK cells were again purified from the co-culture. Cells with high or low proliferative activity were sorted according to the brightness of the cell trace dye, and mRNA was isolated to perform whole genome microarray analysis (fig. 2; red: non-cultured NK cells; green: high proliferative activity; blue: low proliferative activity).

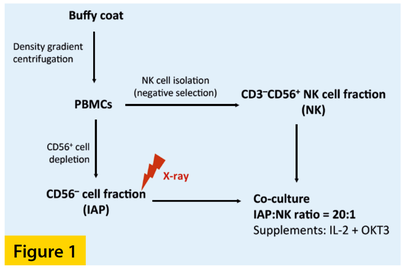



Figure 1

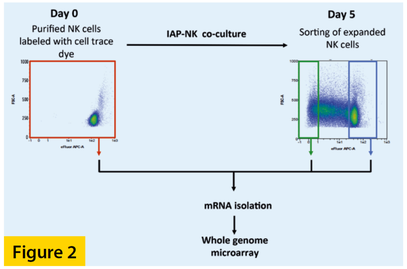



Figure 2

The autoMACS® Pro Separator provides a fully automated cell separation protocol for the isolation of NK cells. PBMCs are first automatically labeled using the NK cell isolation kit, human, after which the cells are separated with an optimized separation protocol. The cells are fully viable and can be used for cell culture, flow analysis or functional assays.

Figure 3

## Results

### 1 Enhancement of NK cell proliferation in short-term culture using autologous irradiated and activated PBMCs

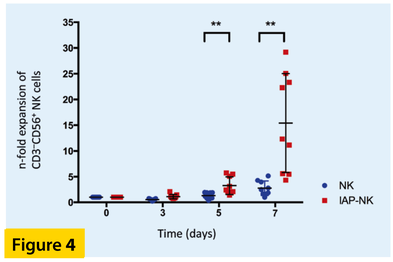



Figure 4

NK cells co-cultured with autologous IAP (IAP-NK) reached a significantly higher expansion rate compared to the same NK cells cultivated with IL-2 supplement only (NK), already after 5 days. Therefore, this time point was selected to perform gene expression analysis of cells showing high or low proliferative activity. Mean values are displayed; $P < 0.05$ was considered significant (paired Student´s t-test).

### 2 Differential gene expression signatures among non-cultured, proliferating, and non-/low proliferating NK cells

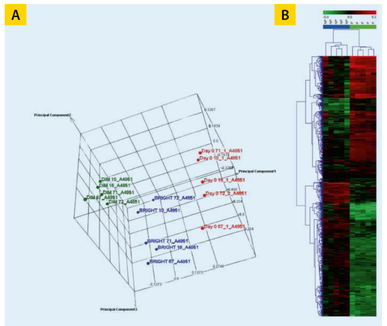



Figure 5

Principal component analysis (PCA) of gene expression profiles showed clearly distinct transcriptional signatures among the three groups of samples (A). The 3D PCA is shown for samples from 5 different donors. Day 0 refers to non-cultured cells, dim and bright refers to proliferating cells and to non-/low proliferating cells, respectively. Differentially expressed transcripts were identified by filtering for statistical relevance and reliable signal intensities (B). The heat map shows the hierarchical clustering analysis of transcripts differentially expressed between proliferating (P) and non-/low proliferating (NP) cells.

### 3 Analysis of the transcripts differentially expressed between proliferating and non-/low proliferating NK cells

Differentially expressed transcripts were classified into different biological processes and pathways according to curated databases (Entrez). Statistical analysis of the gene transcripts associated with the respective categories was also performed (Fisher-Test with Benjamini-Hochberg correction). Figure 6 shows some of the categories containing transcripts with significant up- or down-regulation between proliferating (P) and non-/low proliferating (NP) cells (A) and an example of hierarchical cluster analysis of transcripts related to one of the categories and selected transcripts with differential expression (B).

A

| | Biological processes | Pathways |
|---|---|---|
| Up-regulated transcripts (P vs. NP) | Innate immunity | Induced by IL-2 pathway |
| | Receptor signaling | Induced by TCR pathway |
| | Cell proliferation | Cytokine/chemokine signaling |
| | Cell migration | TCR signaling pathway |
| | Cell differentiation | Antigen presentation |
| | Kinase/phosphatase signaling | Repressed by TGF-β receptor pathway |
| | SAP kinase signaling | |
| | Cell death | |
| Down-regulated transcripts (P vs. NP) | Innate immunity | Repressed by IL-2 pathway |
| | Receptor signaling | Repressed by TCR pathway |
| | Cell proliferation | Cytokine/chemokine signaling |
| | Cell migration | Death receptor signaling |
| | Cell adhesion | |
| | Kinase/phosphatase signaling | |
| | Cell differentiation | |
| | Protein degradation | |

B

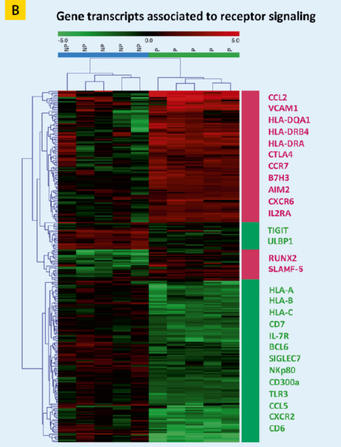
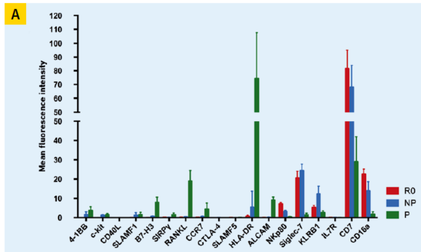



Figure 6

### 4 Validation of expression of selected candidate gene transcripts at the protein level

Some candidate transcripts encoding proteins that are relevant for NK cell biology were selected for further analysis. Protein expression was detected by flow cytometry for most of the candidates. Protein expression levels correlated with the corresponding transcript levels in the respective groups of samples (R0: non-cultured cells; P: proliferating cells; NP: non-/low proliferating cells).

A




B

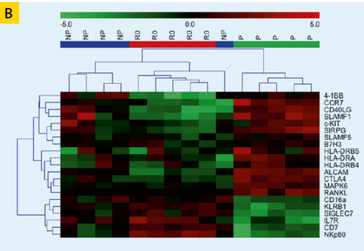



Figure 7

## Conclusion

- We established a co-culture system to expand NK cells *ex vivo*, enabling a microenvironment with multiple signals, appropriate to study early events modulating NK cell proliferation.
- Proliferating and non-/low proliferating NK cells after 5 days of culture show different gene expression signatures.
- NK cells that are actively proliferating after 5 days of culture are characterized by a preferential expression of, for example, RANKL and B7-H3, high levels of HLA-DR alleles, and reduced levels of CD16a, Siglec7, NKp80, and CD7 compared to their non-/low proliferating counterparts.
- Further studies on the identified biomarkers differentiating proliferating and non-/low proliferating NK cells may help to improve current strategies to expand NK cells *ex vivo* for clinical applications.

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