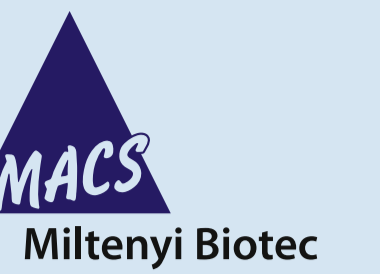


# The capability of CD8<sup>+</sup> and CD8<sup>-</sup> natural killer cells to lyse target cells after isolation with MACS<sup>®</sup> Technology

Anna Maria Turkiewicz, Andrea Jacobs, Jürgen Schmitz, and Volker Huppert  
Miltenyi Biotec GmbH, Bergisch Gladbach, Germany



## Introduction

CD8<sup>+</sup> and CD8<sup>-</sup> NK cells have different capacities to lyse target cells; CD8<sup>+</sup> NK cells have a higher cytotoxic potential compared to their CD8<sup>-</sup> counterpart. Due to the ligation of CD8 on the NK cell surface, CD8<sup>+</sup> NK cells remain protected against effector cell apoptosis and are capable of sequential lysis of multiple target cells (Addison *et al.*, Immunology 2005). Using magnetic cell sorting, the CD8<sup>+</sup> and CD8<sup>-</sup> NK cells were enriched with high purities and recoveries. The two subsets of 10 healthy donors were evaluated in a flow cytometry-based cytotoxicity assay with K562

cells as target cells. After co-culture of NK cells and K562 cells, the NK cells were analyzed by flow cytometry for their extracellular expression of NKp30, NKp44, NKp46, NKG2D, and LAMP-1 as well as their intracellular expression of perforin and granzyme B. Additionally, we intended to evaluate the impact of the CD8 $\alpha$  antibody BW135/80, which is used for cell isolation, on NK cell activation by gene expression profiling using the PIQOR<sup>™</sup> microarray technology, as there was no activation observed by flow cytometric analysis of CD69 expression.

## Methods

CD8<sup>+</sup> and CD8<sup>-</sup> NK cells from 10 different healthy donor PBMCs were isolated using the CD56<sup>+</sup>CD8<sup>+</sup>/CD8<sup>-</sup> NK Cell Isolation Kit. The isolation of the NK subsets was performed in a two-step separation procedure. In a first step, non-NK cells are labeled with a biotinylated antibody cocktail and Anti-Biotin MicroBeads to be magnetically depleted. In a second step, cells are labeled with CD8 MicroBeads to isolate the CD8<sup>+</sup> NK cells by positive selection and to enrich CD8<sup>-</sup> NK cells by depletion of the CD8<sup>+</sup> cells, respectively. Subsequently, cells were analyzed by flow cytometry for the expression of CD3, CD56, and CD8.

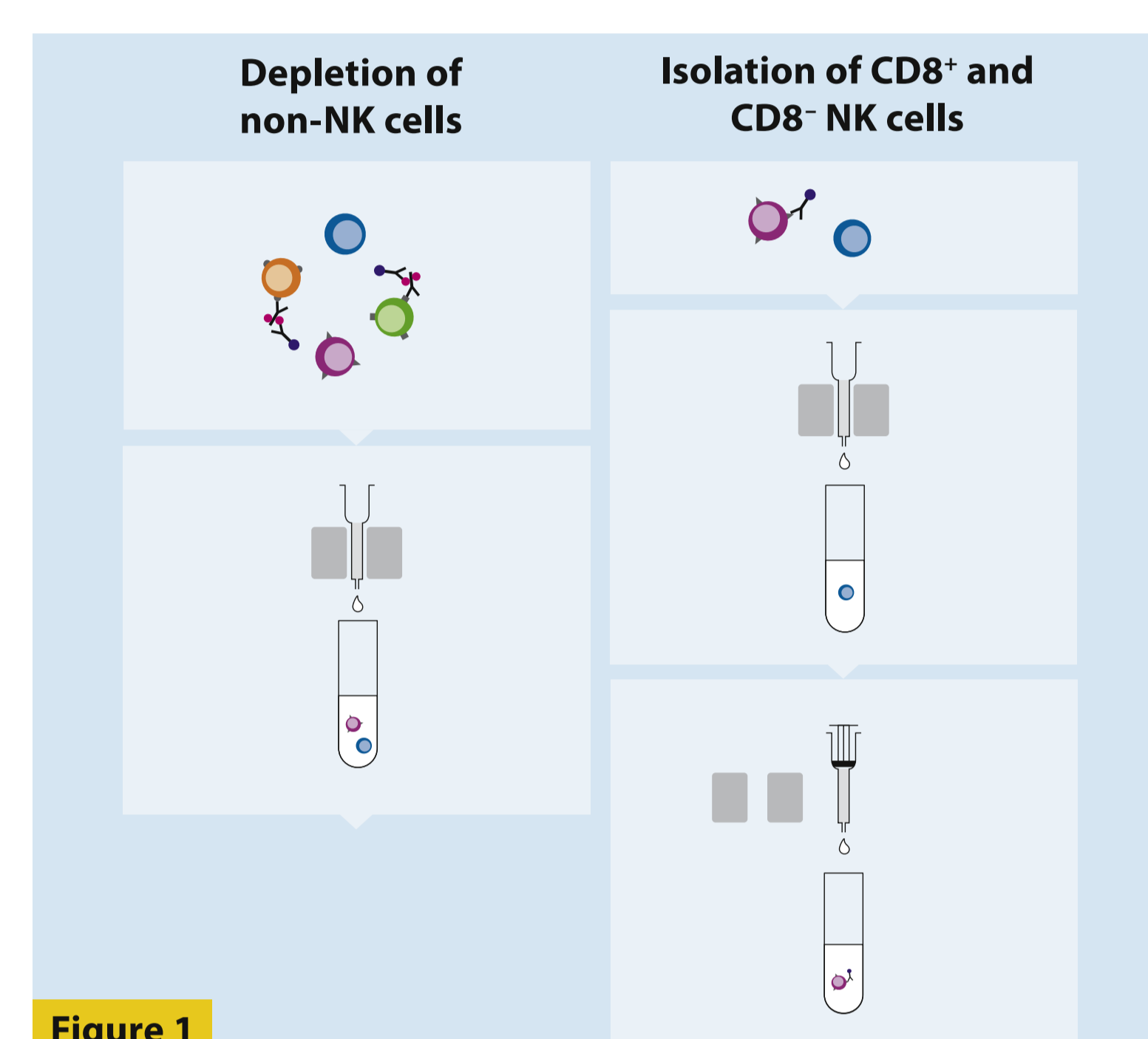


Figure 1

## Cytotoxicity assay

After separation of the two NK cell subsets, a cytotoxicity assay with a GFP-transfected hematopoietic malignant cell line K562 was performed. The optimal effector:target cell ratio and the optimal

time point for measurement of the killing capacity was determined previously. Figure 2 shows the killing capacity of CD8<sup>+</sup> and CD8<sup>-</sup> NK cells of two donors after 4 h assay.

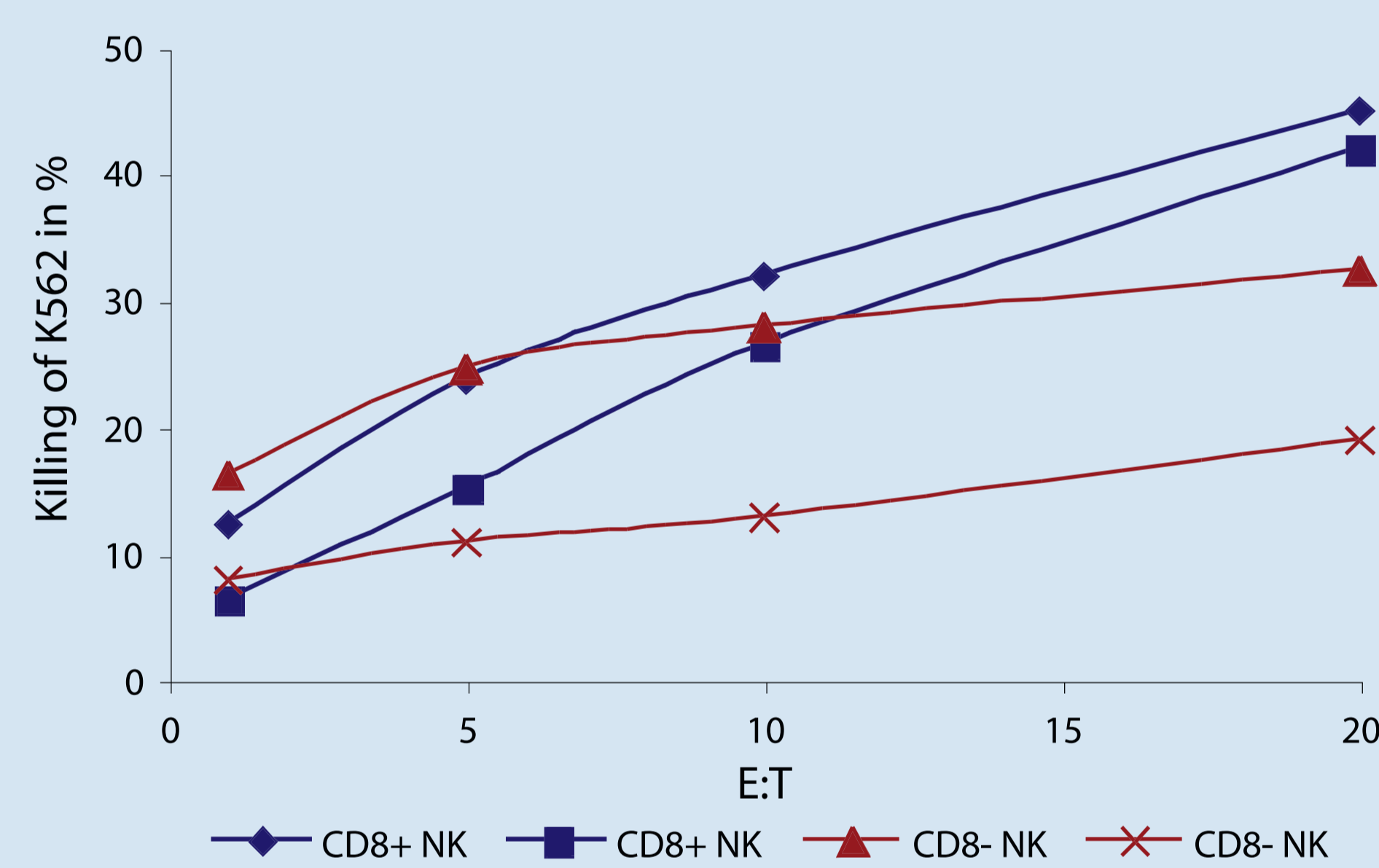


Figure 2

Due to these results, the following assays were performed with a effector:target ratio of 10:1 and 4 h of co-culture. The NK cells and K562 cells were cultivated at a concentration of 10<sup>6</sup> cells/mL in

RPMI + 10% FCS at 37 °C and 5% CO<sub>2</sub>. The killing capacity was evaluated in a flow cytometry-based cytotoxicity assay using propidium iodide.

## Phenotyping

After the co-culture with K562 cells, the cells were phenotypically characterized by flow cytometric analysis. Therefore, NK cells were fixed with formaldehyde and stained with fluorochrome-conjugated antibodies to NKp30, NKp44, NKp46, NKG2D (Miltenyi Biotec). The cells were also

intracellularly stained for the cytotoxins perforin and granzyme B. In order to detect cytolytic activity, fluorescently labeled anti-LAMP-1 antibody was directly added to the co-culture and the cells were washed before analysis.

## Results

### 1 Distribution of CD8<sup>+</sup> and CD8<sup>-</sup> NK cells in human PBMCs

The frequency of the two NK cell subsets was analyzed in ten PBMCs from different donors. The frequencies of CD8<sup>+</sup> NK cells ranged from 2.9% to

9.8% (mean 5.9%) and of CD8<sup>-</sup> NK cells from 2.5% to 9.7% (mean 6.3%).

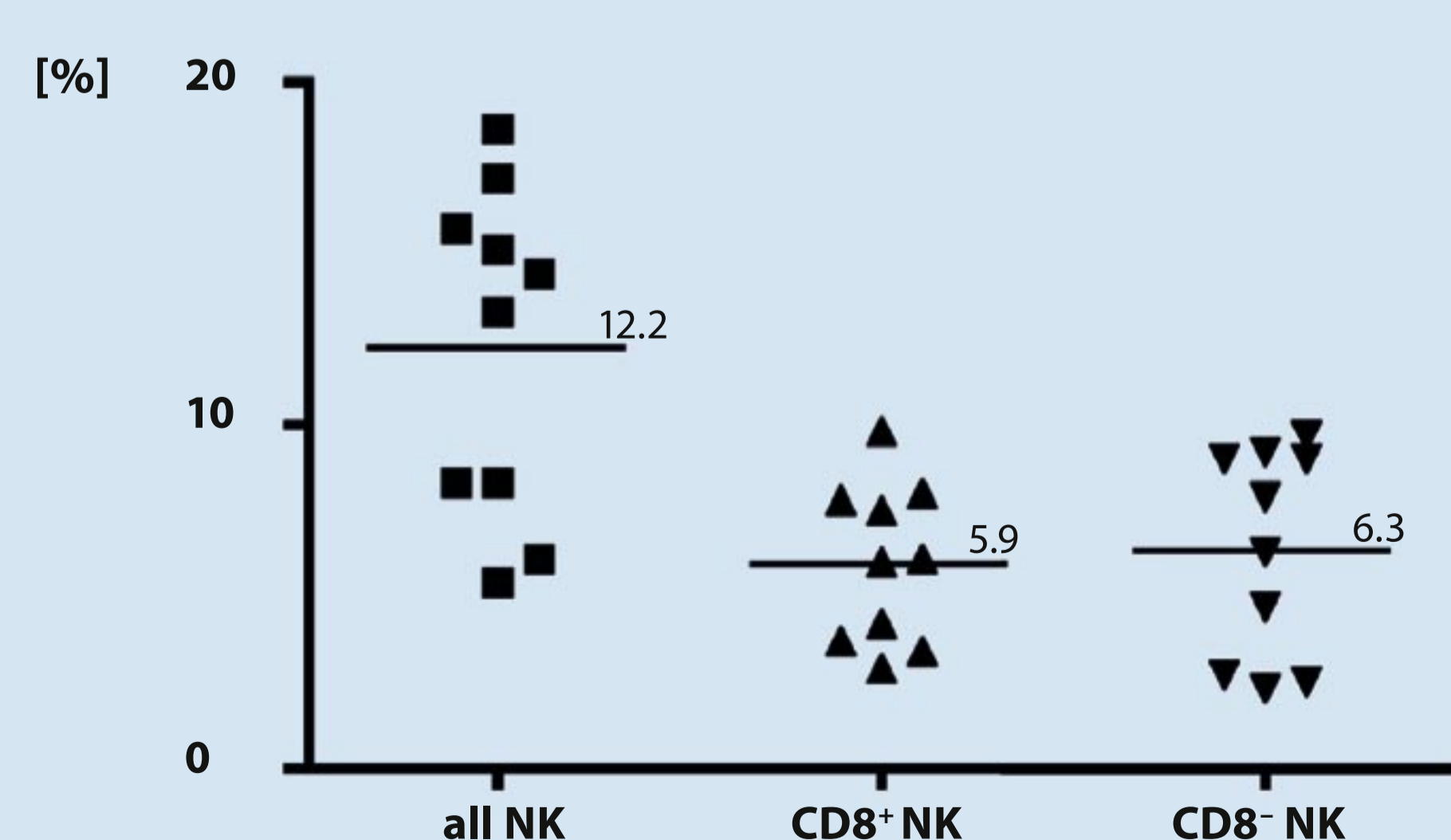


Figure 3

### 2 Isolation of CD8<sup>+</sup> and CD8<sup>-</sup> NK cells

Figure 4 shows a summary of the separation performance for the isolation of CD8<sup>+</sup> and CD8<sup>-</sup> NK cells. The average purity of the CD8<sup>+</sup> subset was 95.9% (range 92.7–97.6%) with a recovery of 58.8% (range 45.8–75.3%). The CD8<sup>-</sup> NK subset showed

an average purity of 94.1% (range 86.6–97.9%) with a recovery of 43.0% (range 24.2–54.7%). Figure 5 shows a typical result for the CD56<sup>+</sup>CD8<sup>+</sup>/CD8<sup>-</sup> NK Cell Isolation Kit.

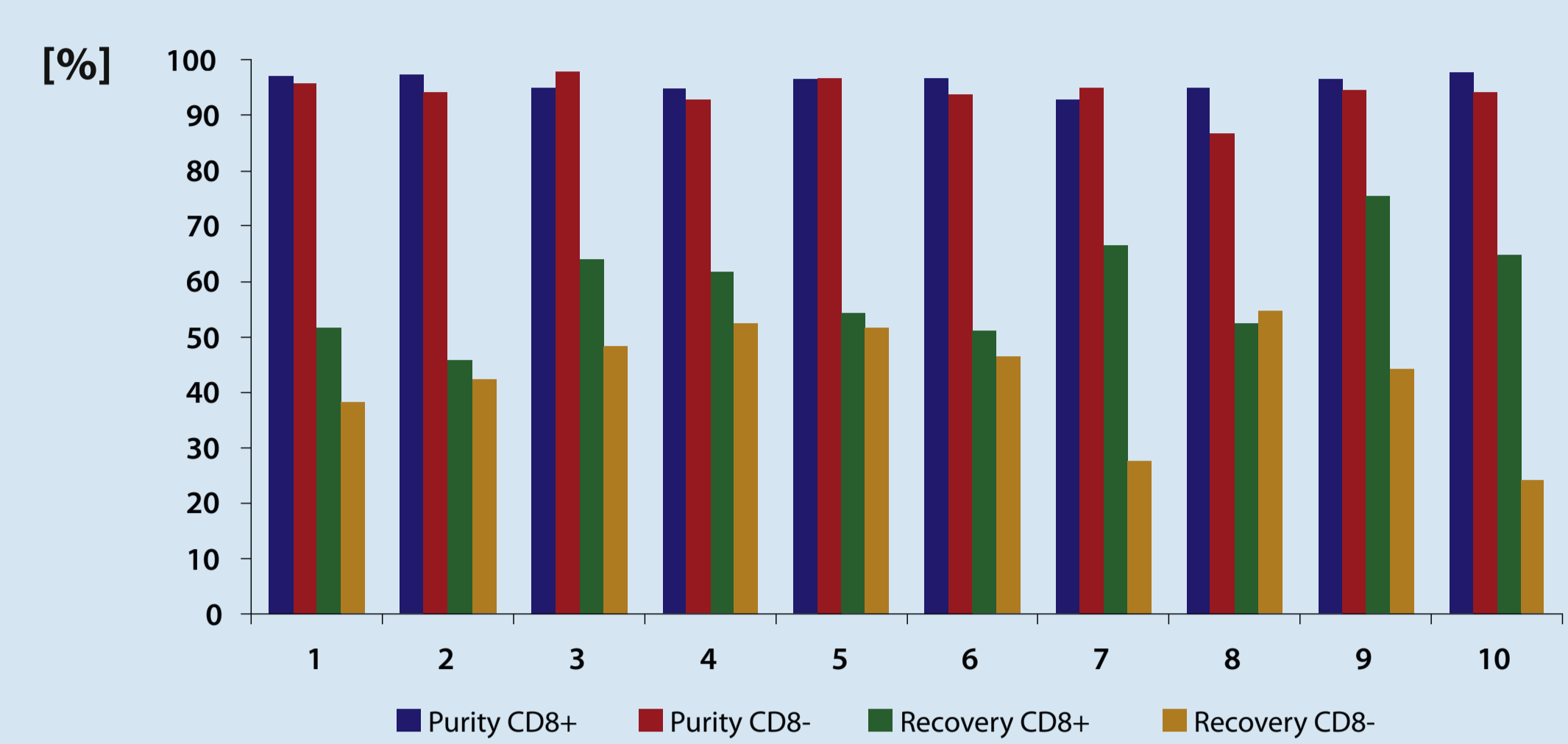


Figure 4

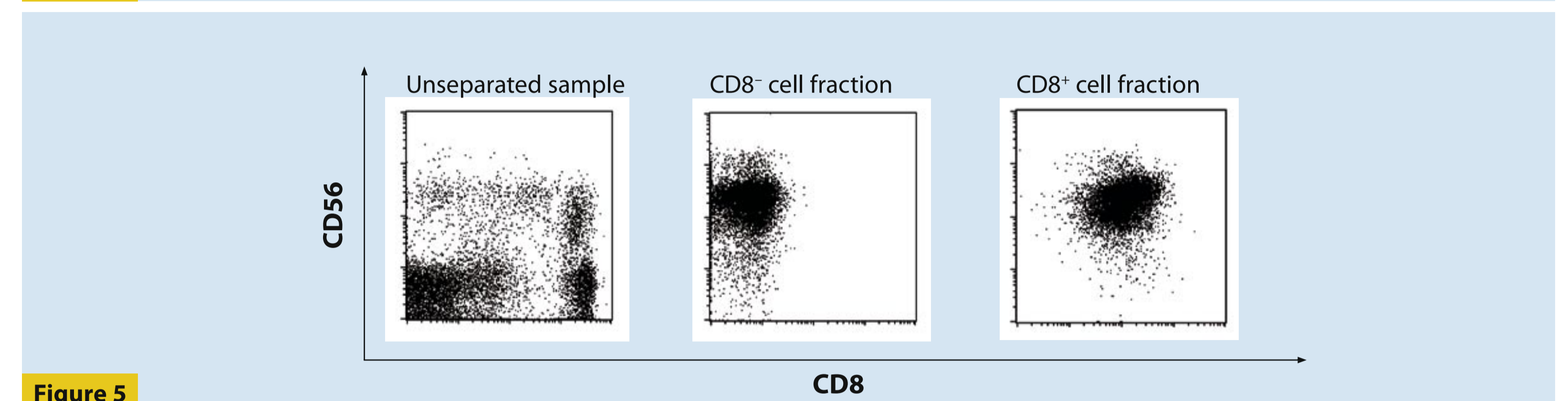


Figure 5

### 3 Cytotoxicity assay

In all ten donors the CD8<sup>+</sup> subset showed significant (P = 0.003) higher lytic capability than the CD8<sup>-</sup> counterpart. Interestingly, the killing capacity of the whole NK cells ranged at the same level compared with the CD8<sup>-</sup> NK cells alone. Figure 6 and table 1 provide an overview of the results.

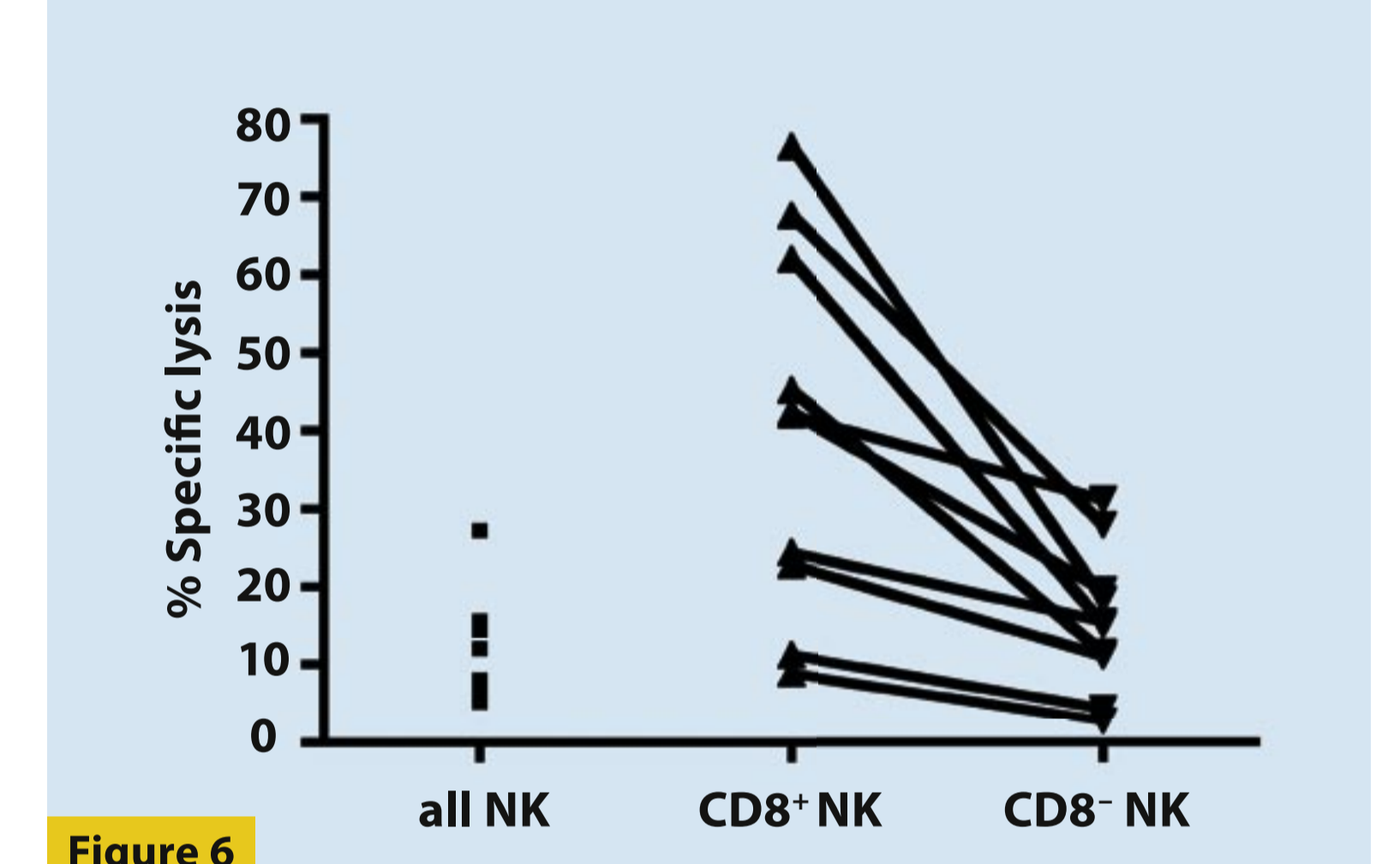


Figure 6

in %	all NK	CD8 <sup>+</sup> NK	CD8 <sup>-</sup> NK
Mean	11.6	40.3	15.9
Median	11.6	41.9	15.8
Range	5.4–27.3	9.0–76.4	3.0–31.4

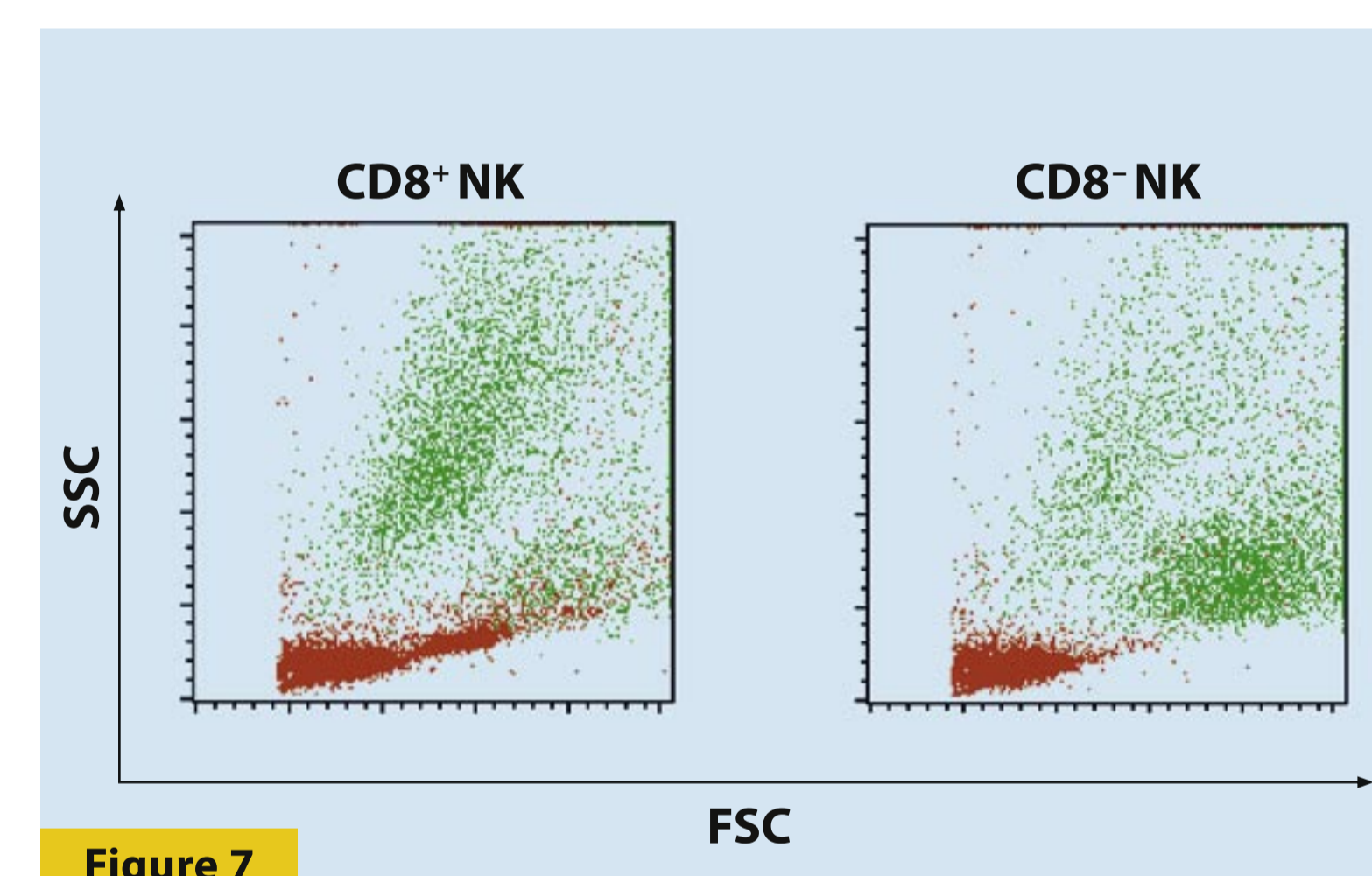


Figure 7

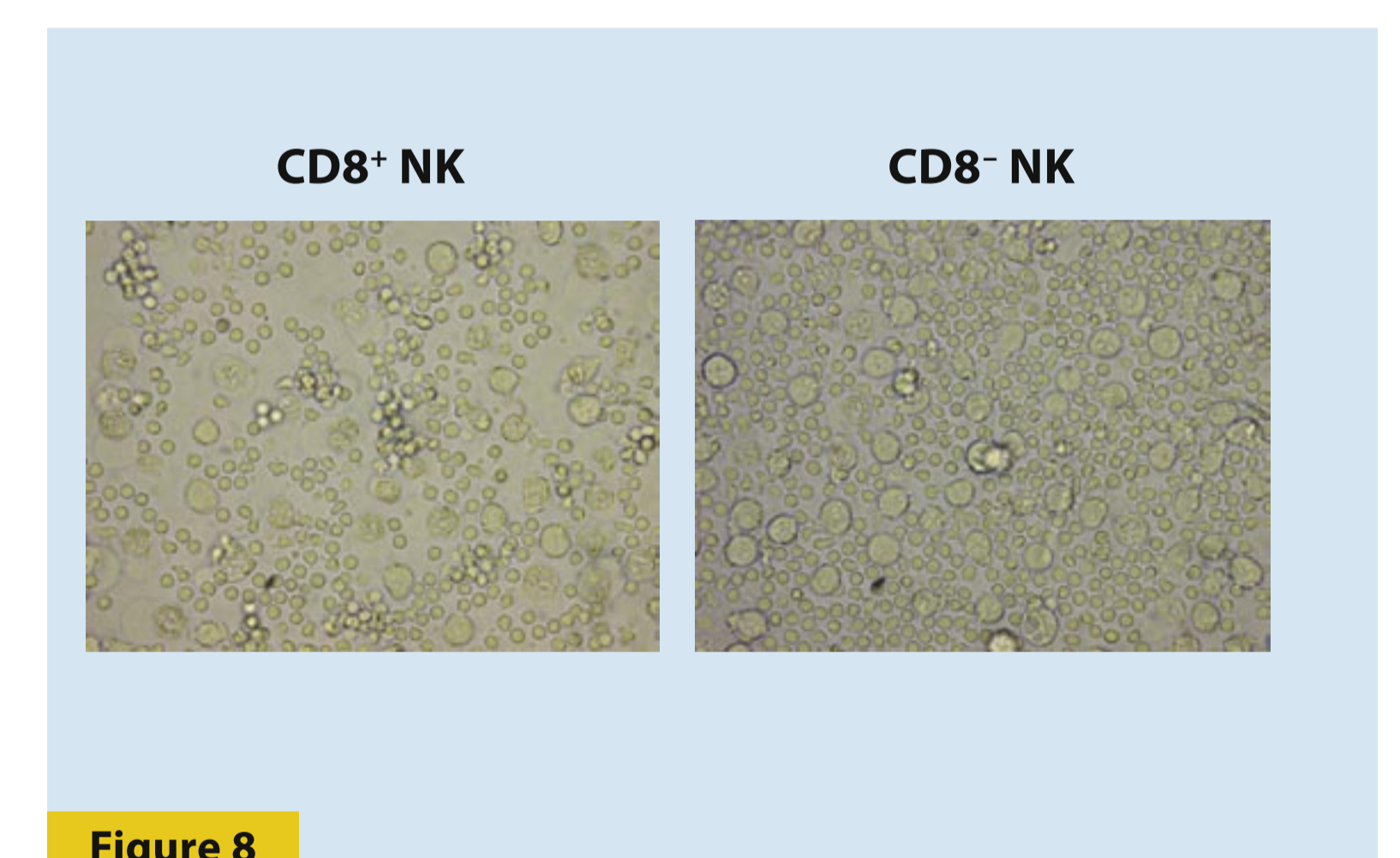


Figure 8

Images of the CD8<sup>+</sup> (left) and CD8<sup>-</sup> (right) NK cells, co-cultured with K562 cells, were taken after 4 h co-culture (fig. 8). The corresponding dot plots (fig. 7) are shown (green dots = K562 cells; red dots = NK

cells). Ligation of CD8<sup>+</sup> NK cells with other NK cells can be observed by microscopy as well as by flow cytometry (formation of doublets and triplets).

### 4 Phenotyping

Staining of aliquots after 4 h of co-culture with NKp30, NKp44, NKp46, NKG2D, perforin, and granzyme B resulted in no significant difference between the CD8<sup>+</sup> and CD8<sup>-</sup> NK cells. Although there was a tendency towards higher expression of LAMP-1 on CD8<sup>+</sup> NK cells, this difference was not significant

(P = 0.098) and there was no correlation between LAMP-1 expression and specific target cell lysis. Figure 8 shows anti-LAMP-1 staining for all NK cells, cultured without K562 cells as control, and CD8<sup>+</sup> and CD8<sup>-</sup> NK cells, co-cultured with K562 cells.

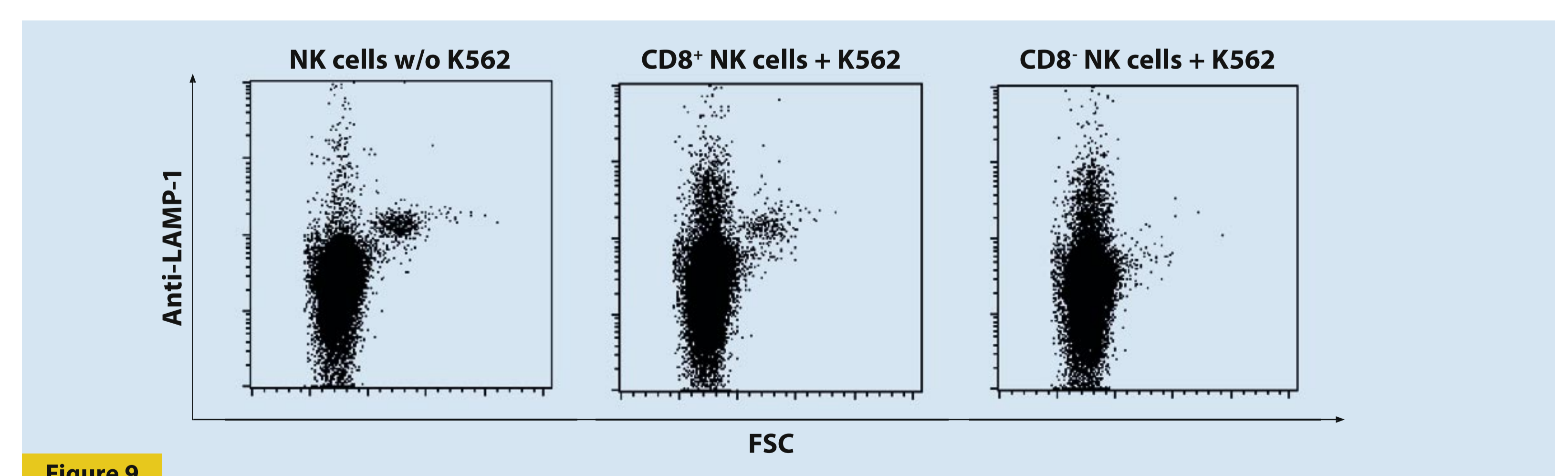


Figure 9

## Conclusion

Using the CD56<sup>+</sup>CD8<sup>+</sup>/CD8<sup>-</sup> NK Cell Isolation Kit, CD8<sup>+</sup> and CD8<sup>-</sup> NK cells of ten different donors (PBMCs) were isolated yielding in high purity and recovery. In cytotoxicity assays with the cell line K562 as target cells, the magnetically separated CD8<sup>+</sup> and CD8<sup>-</sup> NK cells showed effective target cell lysis. As previously reported, the CD8<sup>+</sup> NK cells showed a significant higher cytotoxic capacity compared to their CD8<sup>-</sup> counterpart. Interestingly, there was no significant difference between the two NK subsets regarding the expression of typical NK cell markers NKG2D, NKp30, NKp44, and NKp46, neither in secretion of the cytotoxins such as perforin and granzyme B and no difference in the expression intensity of LAMP-1. The two subsets behaved differently regarding the

ligation of cells as observed by microscopy and flow cytometry. As reported by Addison *et al.*, this ligation of CD8<sup>+</sup> NK cells via the CD8 $\alpha$  molecule leads to a consistent influx of extracellular calcium, which protects the CD8<sup>+</sup> NK cell from apoptosis after target cell lysis. Looking forward, we intend to evaluate the impact of the CD8 $\alpha$  antibody BW135/80, which is used for isolation of CD8<sup>+</sup> cells, on NK cell activation based on gene expression profiling using the PIQOR<sup>™</sup> microarray technology, as there was no activation detectable by flow cytometric analysis of CD69 expression.