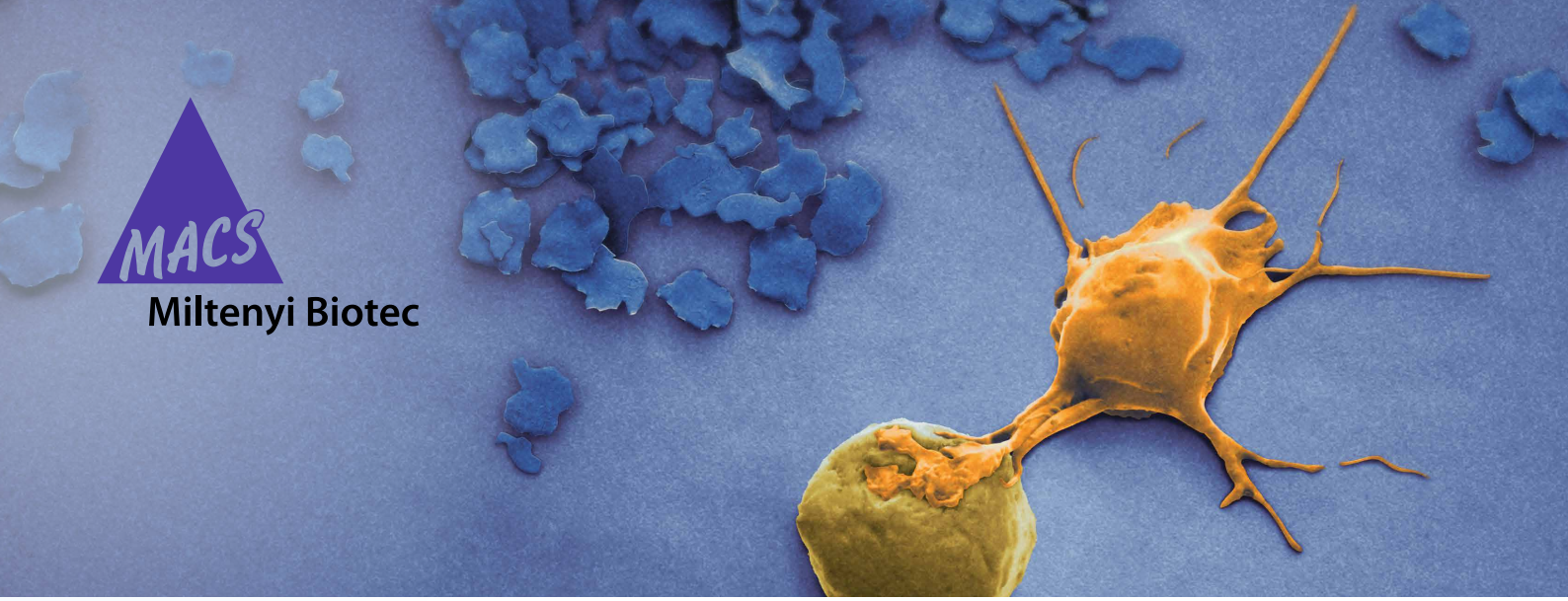

Automated isolation of CD56^{+} natural killer cells and antibody-dependent cellular cytotoxicity

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Background

Natural killer (NK) cells are regulated by a sophisticated system of inhibitory and stimulatory receptors, as well as comodulating receptors. NK cells are characterized by the expression of CD56 and the absence of CD3. They are able to kill target cells via direct cytotoxicity, secretion of cytokines and antibody-dependent cellular cytotoxicity (ADCC) via CD16. CD16 expression is found in a subset of NK cells, CD56^{dim}, and other innate immune cells such as mast cells, macrophages and monocytes[1].

ADCC is a mechanism mediated by NK cells that are activated by antibodies[2]. Antibodies bind antigens on the target cell surface, which is thereby recognized by the CD16 receptor of the NK cells, lysing the target cells upon release of cytotoxic factors[3].

NK cells can be directed and regulated by using highly specific antibodies that recognize cell surface markers of malignant cells with high affinity. Therapeutic antibodies against several targets are commercially available and used in a wide variety of indications[4]. Rituximab for example, targets CD20, an antigen expressed in the cell line Raji. Therefore, the acknowledgement of NK cells as major players in cancer immunotherapies has continually increased in recent years[5].

For ADCC experiments NK cells can be isolated from a variety of starting materials, including peripheral blood mononuclear cells (PBMCs) or directly from blood products like peripheral blood, leukoreduction system chambers (LRSCs), buffy coats or Leukopaks®. Using the lactic acid dehydrogenase (LDH) assay, the cytotoxicity of cells can be quantified by measuring the amount of enzyme released from lysed target cells[3]. Likewise, a killing assay can also be measured using flow cytometry.

Materials and methods

Isolation and cell analysis of NK cells for flow cytometric analysis

NK cells from 40 mL of buffy coat were isolated with StraightFrom® Buffy Coat CD56 MicroBead Kit, human according to manufacturer's instructions. The remaining buffy coat was used to generate PBMCs using density gradient centrifugation to later isolate NK cells with the NK Cell Isolation Kit, human as per manufacturer's instructions.

Staining cocktail
FcR Block, human
Anti-CD45-VioBlue®, human
Anti-CD3-VioGreen™, human
Anti-CD56-VioBright™ 515, human
Anti-CD14-APC-Vio® 770, human
Anti-CD19-APC-Vio 770, human
Anti-CD16-PE, human
Anti-CD25-PE-Vio 770, human
Anti-CD69-APC, human

Table 1: Staining cocktails for flow analysis.

Staining cocktails for the analysis of isolated cells were prepared according to table 1. The individual amounts of fluorochrome-conjugated antibodies are specified in the corresponding antibody data sheets. NK cells were resuspended in the staining cocktail and incubated for 10 min, protected from light, at 2–8 °C. Cells were then washed and resuspended in buffer for analysis using the MACSQuant® Analyzer 10 Flow Cytometer with Flowlogic™ Software.

Isolation of NK cells for LDH assay

NK cells were isolated from buffy coat (Finnish Red Cross Blood Service, Helsinki) according to the manufacturer's instructions with the MultiMACS™ Cell24 Separator Plus and StraightFrom

Buffy Coat CD56 MicroBead Kit, human. Figure 1 shows the basic principle of magnetic cell separation with StraightFrom MicroBeads and the MultiMACS Cell24 Separator Plus. After isolation, NK cells were harvested by centrifugation (200 × g, 7 min) and cell yield was counted using Moxi™ Z Mini Automated Cell Counter (ORFLO® Technologies).

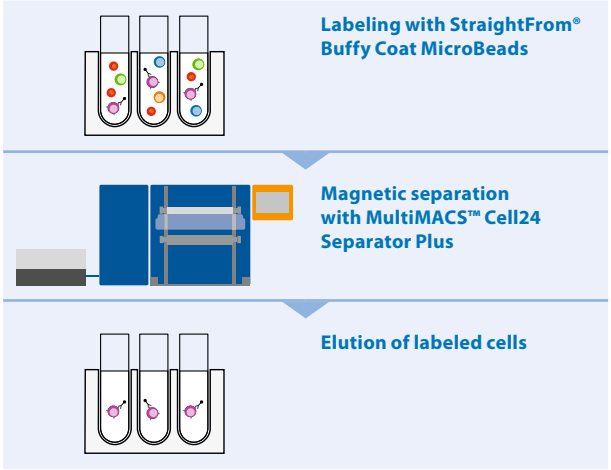

Figure 1: Automated cell isolation with the MultiMACS™ Cell24 Separator Plus

Cell lines and cell culture
For flow cytometric analysis, both Raji and K562 cell lines (target cells) were cultured using RPMI media supplemented with 10% FBS, and Raji cell lines also cultured with 2mM L-Glutamine. NK cells were cultured using NK MACS® medium (NK MACS Basal Medium was mixed with NK MACS Supplement), 5% human AB serum and 500 IU/mL.

For LDH assays, target cell line SK-BR-3 cells (ATCC, #HTB-30) were maintained in DMEM medium supplemented with 10% FBS at 37 °C in a 5% CO_2 cell incubator. NK cells were resuspended in 5% FBS/RPMI 1640 medium after isolation.

Killing assay for flow cytometric analysis
Target cells were labeled with CellTrace™ Violet. Rituximab was added to target cells in concentrations: 20 ng/mL, 100 ng/mL, 500 ng/mL, 1,000 ng/mL and 2,500 ng/mL. Cells were distributed into a 96-well plate. NK cells were added to wells according to two different effector : target ratios (E : T = 1 : 1 and E : T = 5 : 1). After incubation at 37 °C for 3 hours, plates were centrifuged, cells were collected and resuspended in propium iodide (PI) solution before measurement using MACSQuant® Analyzer 10 Flow Cytometer. Data was analyzed with Flowlogic™ Software.

Cytotoxicity assay using LDH
For cytotoxicity testing, target cells were seeded in 96-well plates at a density of 8,000 cells per well and cultured in the presence or absence of test compounds for 4 days. On the day of ADCC testing, the medium and test compound were removed and replaced with 50 µL of 5% FBS/RPMI 1640 medium containing 1 µg/mL trastuzumab (Herceptin®, Roche) or 1 µg/mL omalizumab (Xolair®, Novartis) as nonspecific negative control antibody. The cells were incubated with antibodies for 30 min at 37 °C in a 5% CO_2 cell incubator. NK cells were added (50 µL volume) (E : T = 4 : 1). Antibody treated target cells were incubated with NK cells for 4 hours at 37 °C in a 5% CO_2 cell incubator.

The cytotoxicity assay included the following control treatments. Each was performed in triplicate.

- Target cells + trastuzumab
- Target cells + omalizumab + NK cells
- Target cells + NK cells
- NK cell spontaneous LDH release control
- Target cell spontaneous LDH release control
- Target cell maximum LDH release control
- Culture medium background control

Analysis

After 4 hours of incubation, target cell cytotoxicity was analyzed with the Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific) according to the manufacturer's instructions. Absorption was measured at 490 nm with 680 nm as reference wavelength (A490 nm – A680 nm) using a Multiskan Spectrum plate reader (Thermo Electron Corporation).

The cytotoxicity (%) was calculated using the equation below:

$$\text{Cytotoxicity (\%)} : \frac{\text{Experimental value} - \text{Effector cells SC} - \text{Target cells SC}}{\text{Target cell MC} - \text{Target cells SC}} \times 100$$

MC = maximum control
SC = spontaneus control

Results

NK cells can be isolated directly from buffy coats at high recoveries
The StraightFrom Buffy Coat CD56 MicroBead Kit, human was specifically designed for the separation of NK cells from buffy coats. When isolating NK and natural killer T (NKT) cells with this direct isolation approach and the MultiMACS™ Cell24 Separator Plus for LDH assays, an average yield of $22 \times 10^6 \pm 9.7 \times 10^6$ cells per each half of buffy coat sample were obtained.

NK cells isolated with a StraightFrom isolation strategy (positive isolation) or untouched isolation approach are not activated by the separation
CD56 is expressed on NK cells, but also on NKT cells and some monocyte subsets. Using the StraightFrom CD56 MicroBead Kit, human, enrichment of both NK and NKT cells is achieved in one step from buffy coat, peripheral blood, or LRSC materials. A common misconception regarding positive isolation of NK cells is that this isolation strategy activates the cells. Figure 2 compares isolated NK and NKT cells using positive isolation (StraightFrom Buffy Coat CD56 MicroBead Kit, human) and untouched isolation (NK Cell Isolation Kit, human). Using CD69 and CD25 cell surface markers as indicators of activation, NK cells were not activated and contain the same percentage of $CD16^+$ $CD56^+$ cells.

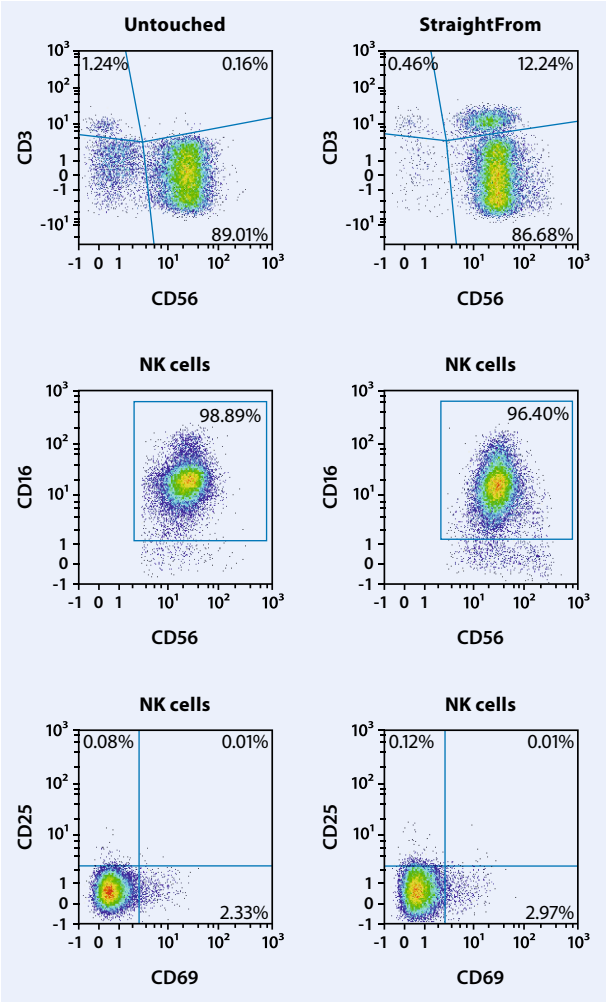

Figure 2: NK cells were isolated via positive selection (right column, StraightFrom CD56 MicroBeads, human) and untouched isolation (left column, NK cell isolation kit, human). Expression of CD16 was determined in $CD3^{-}CD56^{+}$ NK cells as well as CD25 and CD69 were measured as indication of cell activation.

Minimal CD16 expression on NKT cells

StraightFrom® Buffy Coat CD56 MicroBeads allow automated and direct enrichment of NK and NKT cells from buffy coats, without density gradient centrifugation. The cell surface marker CD16 is needed for functional ADCC, and is expressed in monocytes, macrophages, mast cells and NK cells. NK Cells expressed CD16, while NKT cells had minimal expression of CD16 after isolation with StraightFrom Buffy Coat CD56 MicroBeads (figure 3). Therefore, performing ADCC assays with $CD56^{+}$ cells will consequently result in NK cell killing readout without disturbance from NKT cells.

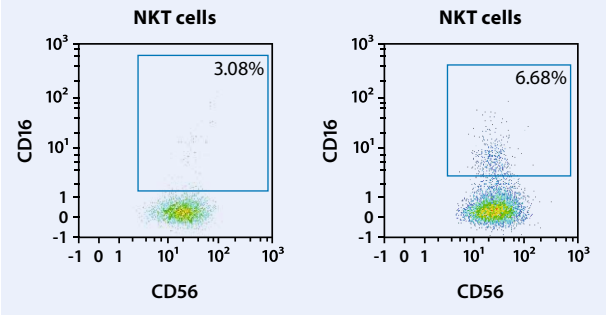

Figure 3: Expression of CD16 on NKT cells after isolation with StraightFrom Buffy Coat CD56 MicroBeads, human. $CD56^{+}CD3^{+}$ were gated on CD16. Figure depicts two different donors.

Antibody dependent cellular cytotoxicity assay using flow cytometry: Similar outcomes using positive or untouched isolation of NK cells

The Raji cell line is generally not susceptible to NK cell killing. However, as observed in figure 4, killing of Raji cell line was enhanced by adding Rituximab. Both isolation strategies, positive using StraightFrom Buffy Coat CD56 MicroBeads or untouched, using NK Cell Isolation Kit, yielded NK cells that show comparable killing using the E : T = 1 : 1 or E : T = 5 : 1. This demonstrates that independently of the isolation strategy and separation product used, NK cells were fully functional and ideally suited for ADCC studies.

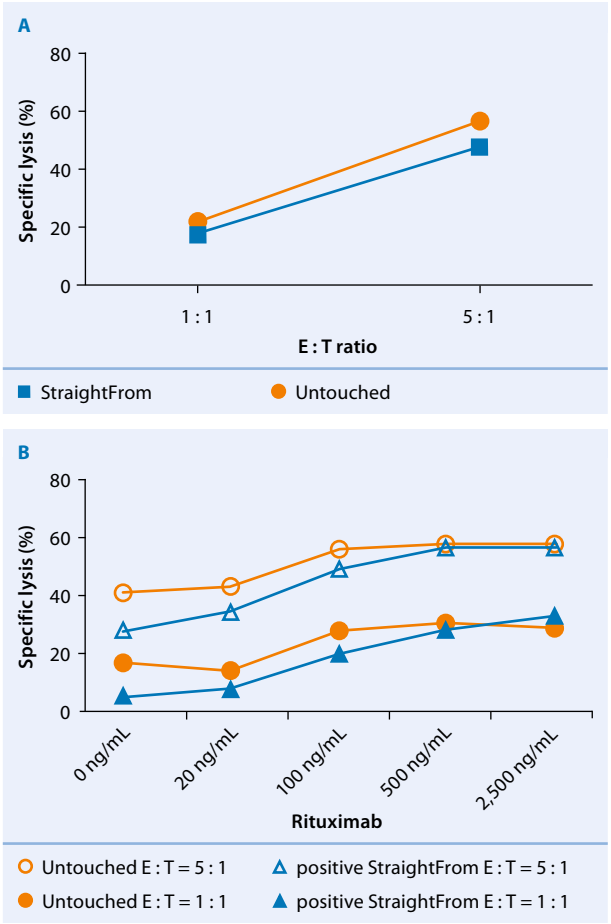

Figure 4: Natural and antibody-dependent cytotoxicity of NK cells isolated via positive or untouched selection. Natural cytotoxicity against cell line K562 was analyzed using NK cells from positive selection or untouched isolations (A). NK cell cytotoxicity against CD20 positive Raji cells was measured using NK cells from positive or untouched isolations. Raji cells were left untreated or treated with 20, 100, 500 or 2500 ng/mL of Rituximab and cocultured with NK cells for 3 h at two different E : T ratios (B).

Antibody dependent cellular cytotoxicity assay using LDH assay

Of all the controls only the lysed target cells showed an elevated absorbance. In addition, this was also observed for target + Herceptin + NK cells, demonstrating that Herceptin is recognized specifically by NK cells, triggering cell death of target cells as expected (figure 5). All other controls did not result in increased absorbance, proving that both the specific antibody for the target cells and the effector NK cells are needed to recognize and kill the target cells.

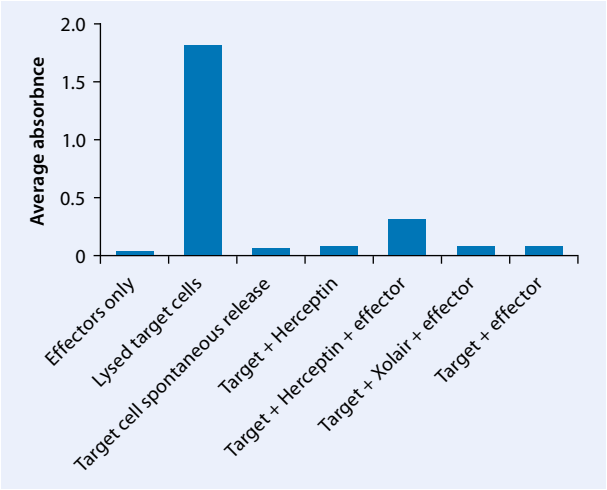

Figure 5: Average absorbance - medium.

For the same passage, the percentage of cytotoxicity was calculated, resulting in 14.2% cytotoxicity when combining target cells, NK cells and Herceptin. All controls showed natural cytotoxicity below 2% (figure 6), demonstrating that cells isolated directly from buffy coat with the StraightFrom® Buffy Coat CD56 MicroBead Kit are fully functional and can be used for ADCC experiments.

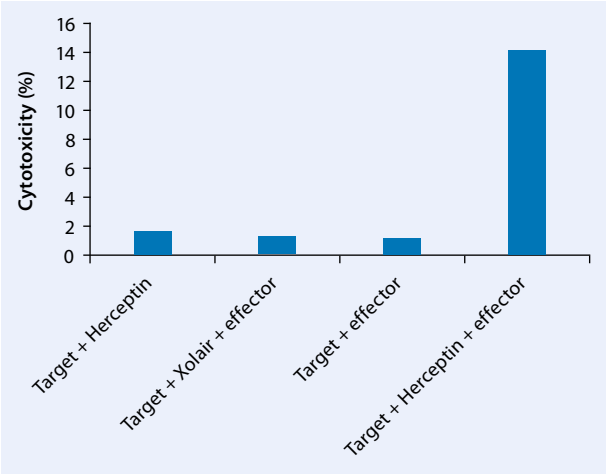

Figure 6: Cytotoxicity (%) of cells in 4 different treatments.

To test the effect of diverse compounds to target cell cytotoxicity, target cells were left untreated or pre-treated with compound A or B for 4 days. Adding each of these compounds enhanced the cytotoxicity of NK cells (figure 7). These results emphasize that a direct, positive isolation approach using StraightFrom Buffy Coat CD56 MicroBeads, yields fully functional NK cells that are suited for ADCC assays, as detected by the LDH detection method.

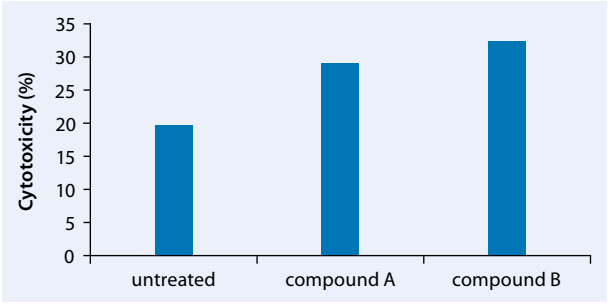

Figure 7: Incubation with compound A and B.

Conclusion

The StraightFrom® Buffy Coat CD56 MicroBead Kit, human and NK Cell Isolation Kit, human provide a suitable solution to isolate viable NK cells at high purities and recoveries, either directly from buffy coat, other blood products or PBMCs.

Negatively isolated NK cells, and even cells isolated with a positive separation approach directly from blood products, are fully functional and perfectly suited for ADCC assays, therefore an untouched isolation approach is not mandatory. As demonstrated, NKT cells do not interfere with ADCC assays, independently of the detection method used.

Automation using the MultiMACS Cell24 Separator Plus, allows for efficient separation of large samples, such as buffy coat, and provides standardized, reproducible results.

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Product	Order no.
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