Automated isolation of CD56\(^+\) natural killer cells and antibody-dependent cellular cytotoxicity

Innate Lymphocytes R&D team, Miltenyi Biotec and Glykos Finland Ltd.

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),uffy coats or Leukopaks\(^6\). 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\(^7\). 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\(^\text{\textregistered}\) 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\(^{\text{TM}}\), human
- Anti-CD3-VioGreen\(^{\text{TM}}\), human
- Anti-CD56-VioBright\(^{\text{TM}}\) 515, human
- Anti-CD14-APC-Vio\(^{\text{TM}}\) 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\(^\text{\textregistered}\) Analyzer 10 Flow Cytometer with Flowlogic\(^{\text{TM}}\) 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\(^{\text{TM}}\) Cell24 Separator Plus and StraightFrom
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 = spontaneous 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 was enriched of both NK and NKT cells 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.
NK 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.
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.

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.

**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.

**References**