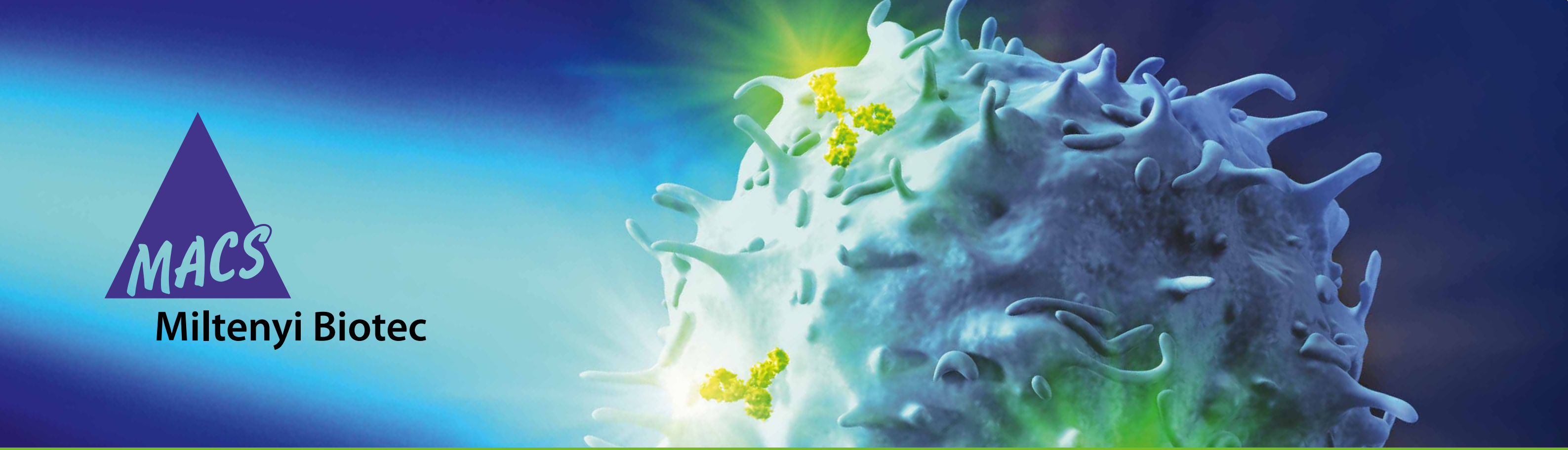

Flow cytometry analysis of whole-blood NK cells expressing single killer cell immunoglobulin–like receptors

Background

Human natural killer (NK) cells are regulated by a sophisticated system of inhibitory and stimulatory receptors as well as comodulating receptors. Killer cell immunoglobulin–like receptors (KIRs) are type 1 transmembrane receptors expressed on NK cells and a subset of T cells. Depending on their structure, KIRs have either inhibitory or activating properties. Inhibitory KIRs are characterized by a long cytoplasmic tail encompassing one or two immunoreceptor tyrosine-based inhibitory motif(s) (ITIM). Expression of both activating and inhibitory KIRs on NK cells occurs randomly. However, the host's genetic environment can influence their expression pattern significantly. Differing expression of KIRs can have effects on the course of various diseases, such as viral infections or autoimmunity, or the outcome of transplantation. For example, KIR ligand mismatching in the graft-versus-host direction has been associated with lower relapse rates as well as better engraftment of T cell–depleted haploidentical transplants.[1,2]

In clinical settings that involve allogeneic NK cells, NK cell alloreactivity has been assessed based on KIR expression and used as tool for optimal donor selection[3]. Several models have been proposed.[4] Models include i) ligand-ligand mismatch[3], ii) receptor-receptor mismatch[5], and iii) receptor-ligand mismatch[6].

Various reports have shown that NK cell alloreactivity can be predicted based on the characterization of KIRs[1,7]. Multicolor flow cytometry is an attractive approach for in-depth phenotypic characterization of NK cells. Moreover, it is the easiest method to quantify NK cells expressing only one inhibitory KIR gene, i.e., cells that are negative for all other MHC-inhibitory receptors[8]. The number of cells expressing only a single KIR is in direct proportion to the killing activity against target cells that do not express the corresponding ligand (receptor–ligand mismatched).
The four main inhibitory KIRs for which ligands have been identified are KIR2DL1, KIR2DL2, KIR2DL3, and KIR3DL1 (fig. 1).

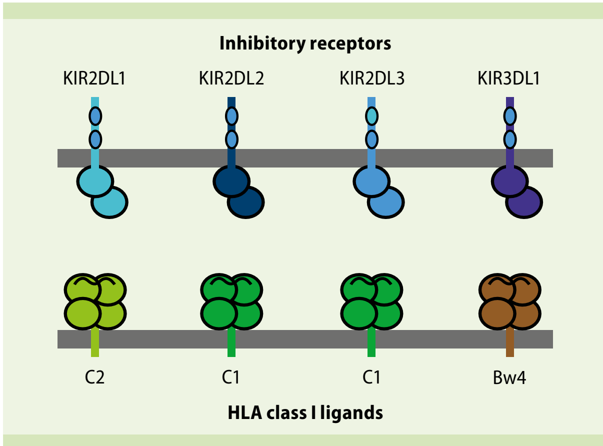

Figure 1: Inhibitory KIRs and HLA class I ligands.

This application note describes the analysis of the human KIR repertoire by flow cytometry. We present an antibody panel allowing simultaneous assessment of the four major inhibitory KIRs (KIR2DL1, KIR2DL2/DL3/DS2, KIR3DL1) and an additional inhibitory NK cell receptor, namely NKG2A.

Materials and methods

Blood samples

Whole blood from ten donors was collected and used within 24 h after collection. Samples were processed as shown in figure 2.

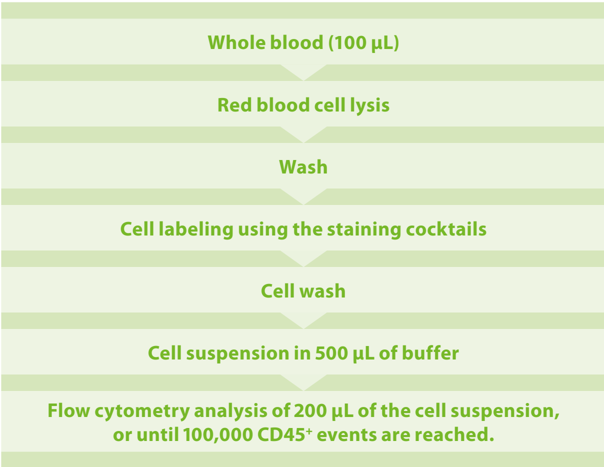

Figure 2: Workflow for the analysis of KIRs and NKG2A receptors in NK cells from whole blood.

Preparation of solutions and staining cocktails

Red Blood Cell Lysis Solution

On the day of NK cell analysis, 2.5 mL Red Blood Cell Lysis Solution (10×) was combined with 22.5 mL of double-distilled water and mixed well. The resulting 1× Red Blood Cell Lysis Solution was stored at room temperature (20–25 °C) until use. Unused solution was discarded.

Buffer

PBS/EDTA Buffer or autoMACS® Rinsing Solution were supplemented with HSA or BSA at a final concentration of 0.5%.

Staining cocktails

Staining cocktails for the analysis of cells expressing single inhibitory receptors were prepared according to table 1. The proper concentrations of the individual fluorochrome-conjugated antibodies are specified in the corresponding antibody data sheets. The final volume of the four cocktails was adjusted to 110 µL using PBS/EDTA Buffer. The signal for the inhibitory receptor of interest was detected in the PE channel of the flow cytometer, whereas the other inhibitory receptors were detected collectively in the APC channel.

Cell staining

KIR2DL1⁺, KIR2DL2/DL3/DS2⁺, KIR3DL1⁺, and NKG2A⁺ cells from whole blood were labeled with fluorochrome-conjugated monoclonal or recombinant antibodies. In summary, four 5 mL tubes were labeled as (1) CD158a (KIR2DL1), (2) CD158b (KIR2DL2/DL3), (3) CD158e (KIR3DL1) and (4) CD159a (NKG2A). Whole blood (100 µL) was added to the tube, and red blood cells were lysed by adding 2 mL of 1× Red Blood Cell Lysis Solution, mixing immediately, and incubating in the dark at room temperature until the suspension was transparent red (10–15 min). After red blood cell lysis, cells were washed in buffer. Subsequently, cells were resuspended in 110 µL of the respective staining cocktail and incubated for 10 min in the dark at 2–8 °C. Cells were then washed and resuspended in 500 µL of buffer for analysis on a MACSQuant® Analyzer 10 using the MACSQuantify™ Software.

Results

Flow cytometry analysis of NK cells that are single-positive for four inhibitory receptors

Blood from ten healthy donors was processed as described in the methods sections. The antibody panels presented allow the flow cytometric determination of NK cells that are single-positive for a given inhibitory receptor. A summary of the percentages of NK cells expressing only one of the four inhibitory receptors is shown in figure 3. Compared to the KIRs, percentages of NKG2A single-positive cells showed greater variation between donors.

Inhibitory receptor of interest	CD158a (KIR2DL1)	CD158b (KIR2DL2/DL3)	CD158e (KIR3DL1)	CD159a (NKG2A)
Single inhibitory receptor staining cocktail	CD158a (KIR2DL1)-PE	CD158b (KIR2DL2/DL3)-PE	CD158e (KIR3DL1)-PE	CD159a (NKG2A)-PE
	–	CD158a (KIR2DL1)-APC	CD158a (KIR2DL1)-APC	CD158a (KIR2DL1)-APC
	CD158b (KIR2DL2/DL3)-APC	–	CD158b (KIR2DL2/DL3)-APC	CD158b (KIR2DL2/DL3)-APC
	CD158e (KIR3DL1)-APC	CD158e (KIR3DL1)-APC	–	CD158e (KIR3DL1)-APC
	CD159a (NKG2A)-APC	CD159a (NKG2A)-APC	CD159a (NKG2A)-APC	–
Backbone staining cocktail	CD3-PerCP-Vio® 700			
	CD14-PerCP-Vio 700			
	7-AAD			
	CD45-VioBlue®			
	CD56-VioBright™ 515			
	FcR Blocking Reagent			
Final volume of the complete cocktail	110 µL			

Table 1: Composition of staining cocktails.

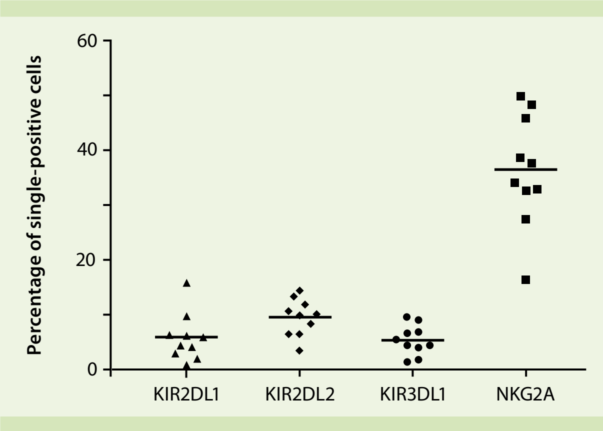

Figure 3: Percentages of NK cells that are single-positive for the indicated inhibitory receptors. Data show results from whole blood samples obtained from ten different donors.

In haploidentical stem cell transplantation for patients with AML, a KIR mismatch between the donor and the host in the graft-versus-host (GVH) direction has been reported to result in a reduced risk for relapse, graft-versus-host disease (GVHD), and graft rejection[1,9-11]. In the context of research towards the development of advanced NK cell therapies, a comprehensive analysis of the KIR repertoire therefore provides highly valuable information.

Figure 4 shows the percentages of cells that were single-positive for KIR2DL1 in whole blood samples from ten different donors. Data indicate that the frequencies of these cells varied between donors. The sample from a donor with large numbers of KIR2DL1⁺ cells is highlighted in pink. According to the literature[1,9-11], this phenotype, for instance, might be preferable for a recipient lacking HLA-C2.

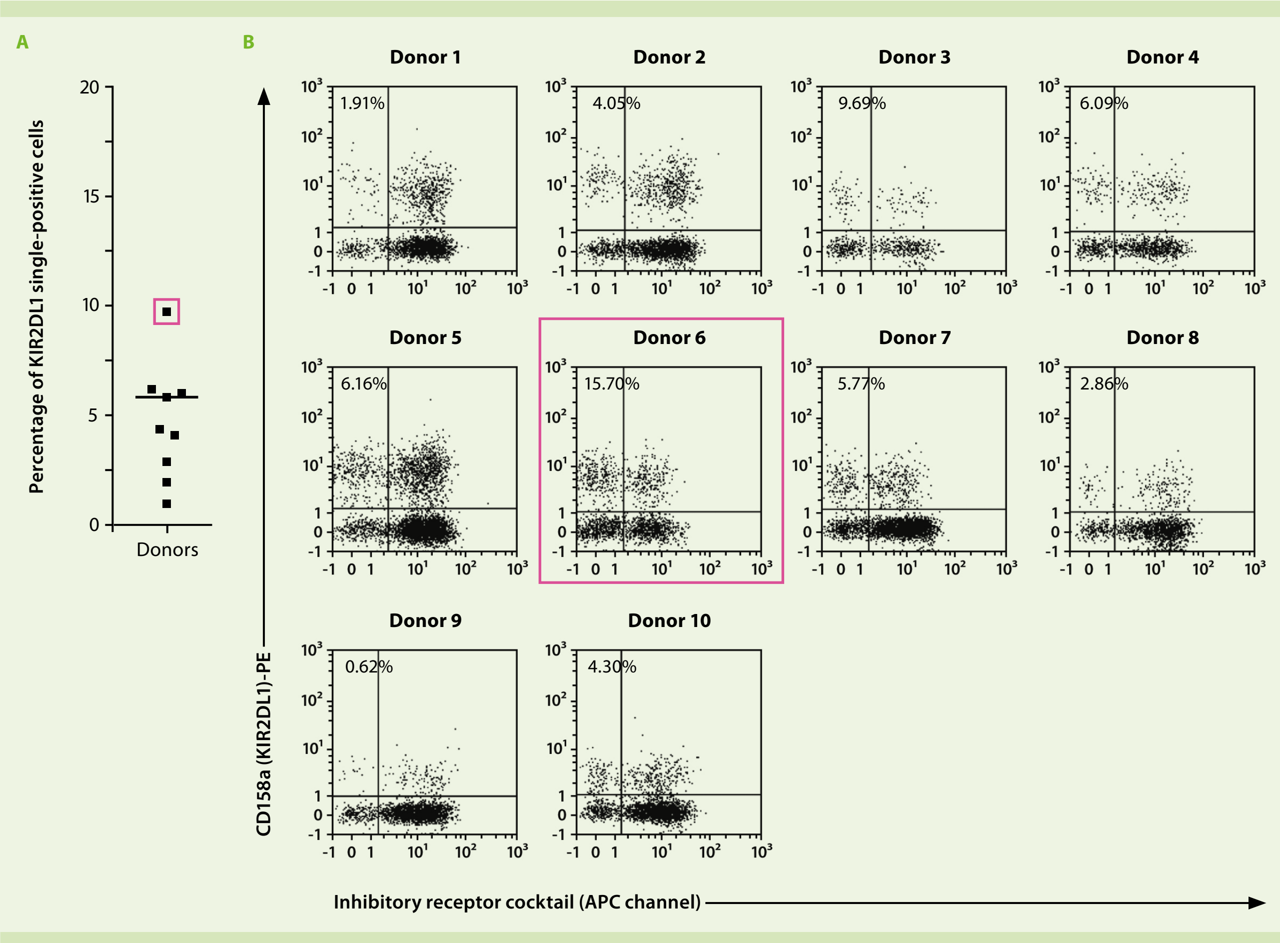

Figure 4: KIR2DL1 single-positive NK cells in ten donors. Peripheral whole blood was stained with the appropriate cocktail described in materials and methods. Cells were analyzed by flow cytometry on the MACSQuant Analyzer 10. Cell debris and dead cells were excluded from the analysis based on scatter signals and 7-AAD staining. NK cells that were single-positive for KIR2DL1 were assessed by gating on i) 7-AAD⁻ live cells, ii) CD3⁻CD56⁺ NK cells, and iii) cells positive for KIR2DL1 and negative for the other three inhibitory receptors.

Conclusion and outlook

- The presented data show the feasibility of evaluating the presence of NK cells that are single-positive for inhibitory receptors in whole blood samples. Flow cytometry analysis is based on four different antibody panels.
- Future work includes the automation of flow cytometry analysis using the MACSQuant® Analyzer 10 and MACSQuantify™ Software.

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Miltenyi Biotec product			Order no.
Antibodies	**Fluorochrome**	**Clone**	
CD56	VioBright 515	REA196	3)
CD45	VioBlue	REA747	3)
CD3	PerCP-Vio 700	REA613	3)
CD14	PerCP-Vio 700	REA599	3)
CD158a (KIR2DL1)1)	PE/APC	REA284	3)
CD158b (KIR2DL2/DL3)2)	PE/APC	DX27	3)
CD158e (KIR3DL1)	PE/APC	DX9	3)
CD159a (NKG2A)	PE/APC	REA110	3)
Reagents and solutions			
FcR Blocking Reagent, human			130-059-901
7-AAD Staining Solution			130-111-568
Red Blood Cell Lysis Solution (10×)			130-094-183
autoMACS Rinsing Solution			130-091-222
Instrument and software			
MACSQuant Analyzer 10			130-096-343 4)
MACSQuantify Software			4)

1) The clone used in this study has been shown to cross-react with KIR2DS5.[12]

2) The clone used in this study has been reported to cross-react with KIR2DS2.[12]

3) These antibodies are available conjugated to a wide range of other fluorochromes. For details and ordering information on MACS Antibodies visit **www.miltenyibiotec.com/antibodies.**

4) For the complete range of flow cytometers and software options visit **www.miltenyibiotec.com/flowcytometry.**

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