Flow cytometric multicolor NK cell phenotyping as a potential tool for donor selection and NK cell reconstitution analysis in different hematopoietic stem cell transplantation setups

Miltenyi Biotec

Introduction

Natural killer (NK) cells are lymphocytes of the innate immune system and show the capacity to kill cancer cells. Their function is regulated by the balance of inhibitory and activating signals transmitted mainly by killer-cell immunoglobulin-like (KIR), killer-cell lectin-like (KLR), and natural cytotoxicity (NCR) receptors.[1] Genotyping, phenotyping, and haplotyping of NK cell receptors are performed in different allogeneic hematopoietic stem cell transplantation (HSCT) settings to correlate donor- and patient-specific NK cell characteristics with the incidence of graft-versus-host disease (GvHD), graft-versus-leukemia effects (GvL), graft rejection, and relapse, with the aim to improve donor selection.[2,3] Furthermore, NK cell receptor phenotyping is a potent tool to analyze NK cell reconstitution after allogeneic HSCT[4] to further improve the understanding of the complex NK cell biology. Here we present four robust 8-color flow cytometry NK cell receptor phenotyping panels applicable to EDTA–whole blood, peripheral blood mononuclear cells (PBMCs), and isolated NK cells. Panels focus on a) inhibitory KIRs, b) other inhibitory and activating KIRs, c) KLRs and NK cell maturation markers, and d) NCRs. Panels include recombinant REAfinity™ Antibodies, which provide the advantages of high purity, lot-to-lot consistency for greater reproducibility, and high specificity, so that FcR blocking is not required. Additionally, the brighter VioBright™ FITC fluorophore is used to substitute conventional FITC in the case of the CD335 antibody. FlowLogic™ Software was used retrospectively for automated compensation of spectral overlap in order to reduce hands-on time.

Methods

Preparation of cellular material

Whole blood from healthy donors was collected using K²EDTA BD™ Vacutainer® Tubes and stained using a lyse/wash procedure followed by flow cytometric analysis. PBMCs were enriched by density gradient centrifugation from buffy coat obtained from healthy donors. While half of the PBMCs were directly stained and analyzed by flow cytometry, the other half was used for the isolation of label-free NK cells (NK Cell Isolation Kit, human) on the autoMACS® Pro Separator, followed by NK cell receptor phenotyping. Results from the different cellular materials were then compared to each other.

NK cell phenotyping by flow cytometry

We established four antibody panels with backbone staining of CD3, CD56, and SYTOX® Blue Dead Cell Stain (Life Technologies) to analyze 24 different NK cell receptors on 100 µL EDTA–whole blood, 5×10^5 PBMCs, or 1×10^5 isolated NK cells. Fluorochrome-conjugated antibodies (Miltenyi Biotec; table 1) for the most relevant NK receptors recognizing HLA class I molecules, including HLA-C1, HLA-C2, HLA-Bw4, and HLA-Cw4 alleles, and other receptors involved in inhibiting and activating NK cell activity were selected with preference for available recombinant REAfinity™ Antibodies (marked in bold) and bright fluorescent dyes (VioBright FITC). The antibody panels were optimized to match the configuration of the MACSQuant® Analyzer 10. Non-specific binding of antibodies was blocked by addition of Tandem Signal Enhancer.

Retrospective compensation of spectral overlap

For retrospective compensation of spectral overlap, single-stained MACS® Compensation Beads (MACS Comp Bead Kit, anti-REA/anti-mouse Igκ) were measured on the MACSQuant Analyzer 10, in addition to multicolor-stained samples. Analysis was done using the FlowLogic Software (version 7).

Compensation of spectral overlap was performed for each panel separately by FlowLogic on single-stained beads in a retrospective manner. For each fluorochrome-conjugated antibody, the main bead population was identified (fig. 2A) and unstained and stained beads were discriminated (fig. 2B). Compensation was executed automatically by the FlowLogic Software for each fluorochome against all other channels (fig. 2C). The overlay of compensation (fig. 2D) showed adequate results for all panels (compensation of the KIR panel 158abe is shown by way of example).

	VioBlue	VioGreen	FITC	VioBright FITC	PE	PerCP-Vio 700	PE-Vio 770	APC	APC-Vio 770
KIR panel CD158abe	CD158e1 SYTOX Blue	**CD3**	**CD158a**	–	**CD158b2**	**CD158e1/e2**	CD158i	CD158b1/b2	**CD56**
Detailed KIR panel	CD158a/h SYTOX Blue	**CD3**	**CD158a**	–	CD158f	**CD158e1/e2**	CD158e/k	CD85j	**CD56**
KLR/ maturation panel	**CD16** SYTOX Blue	**CD3**	CD57	–	CD314	**CD94**	**CD159a**	CD159c	**CD56**
NCR panel	**CD244** SYTOX Blue	**CD3**	–	CD335	CD336	CD8	**CD161**	CD337	**CD56**

Table 1

The autoMACS® Pro Separator (fig. 1) allows walk-away cell isolation with fully automated labeling and separation, resulting in high purity and recovery of target cells. Furthermore, the MACS® column technology together with isolation kits enable true untouched isolation of target cells with minimal labeling. Unlike column-free methods, this approach leaves all target cell epitopes free of labeling and allows for unbiased phenotypic analysis of surface markers using flow cytometry.



Figure 1

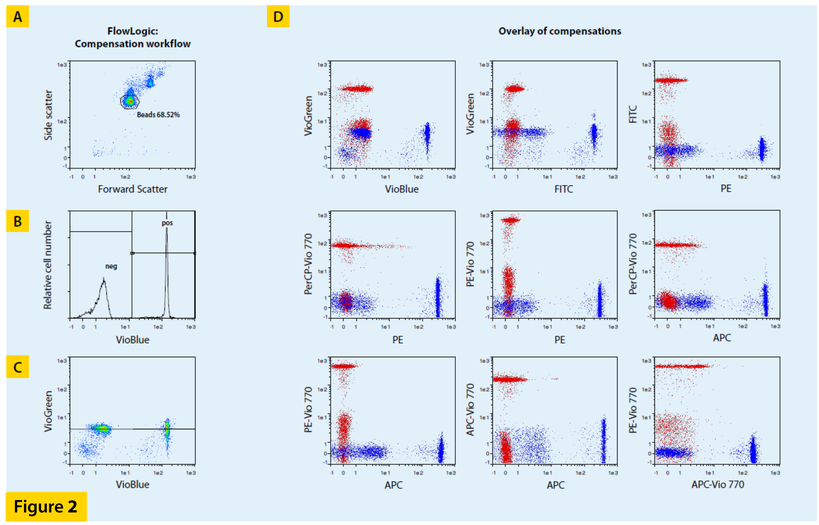


Figure 2

Results

1 Antibody panels for the comprehensive flow cytometric analysis of 24 NK cell receptors

We established four antibody panels with backbone staining of CD3 and CD56 to analyze 24 different NK cell receptors. As an example, figure 2 shows the analysis of PBMCs with these panels. Analysis was also feasible for EDTA-whole blood and isolated NK cells. All samples were measured without compensation of spectral overlap using the MACSQuant Analyzer 10. The compensation matrices were applied retrospectively during analysis with FlowLogic Software. The backbone gating of each antibody panel was based on the exclusion of debris, doublets, dead cells, and CD3⁺ T and NKT cells, as well as gating on CD56⁺ NK cells (fig. 3, left). KIRs, KLRs, and NCRs were analyzed on CD56⁺ NK cells. Where appropriate, $CD56^{dim}$ cytotoxic NK cells and $CD56^{high}$ resting, regulatory NK cells were differentiated during analysis. Some staining patterns were not feasible for isolated NK cells (highlighted in blue), because no marker-negative cell population was detectable for adequate gating. In addition to the gating strategy shown for KIR panel CD158abe, it was possible to identify KIR-negative, CD158a/b/e single-positive NK cells (data not shown). Analysis with the KLR panel/maturation was possible for total NK cells or NK cell subsets discriminated by CD56 and CD16 expression (bottom dot plot). The NCR panel was applicable to total NK cells or NK cell subsets discriminated by CD56/CD8 expression (bottom dot plot).

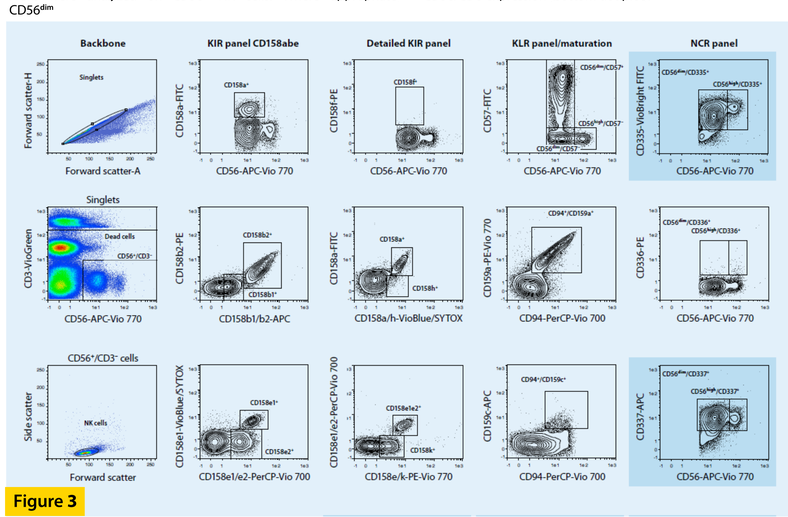


Figure 3

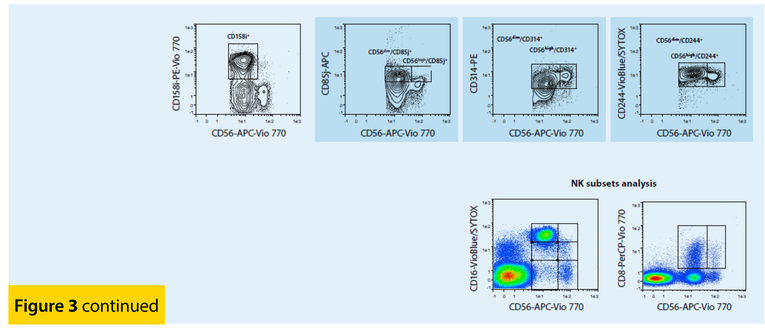


Figure 3 continued

2 NK cell phenotyping of EDTA–whole blood, PBMCs, and isolated NK cell samples from healthy donors

Multicolor flow cytometry was performed on EDTA–whole blood (n = 6) and PBMCs (n = 12) from healthy donors using the above- mentioned antibody panel "KIR panel CD158abe" (fig. 4A, top) to identify KIR-negative, CD158a/b/e single-positive NK cells (fig. 4B, top). Both cell sources showed comparable KIR expression patterns, indicating that both materials were equally usable for multicolor KIR phenotyping. Furthermore, pairs of PBMCs and isolated NK cells from three healthy donors were analyzed in the same way (fig. 4A and B, bottom). Even when different cell populations, e.g., PBMCs vs. isolated NK cells, from the same donor were used, expression levels of NK cell receptors were not significantly different from each other, with regard to the "KIR panel CD158abe" (fig. 4A and B, bottom) and the other three panels (data not shown). Minor, non-significant deviations of marker expression within the range of inter-assay variation (≤10%) were observed on isolated NK cells compared to PBMCs. Data were generated in independent experiments on six different days. Single data points represent samples from individual donors, lines indicate the means.

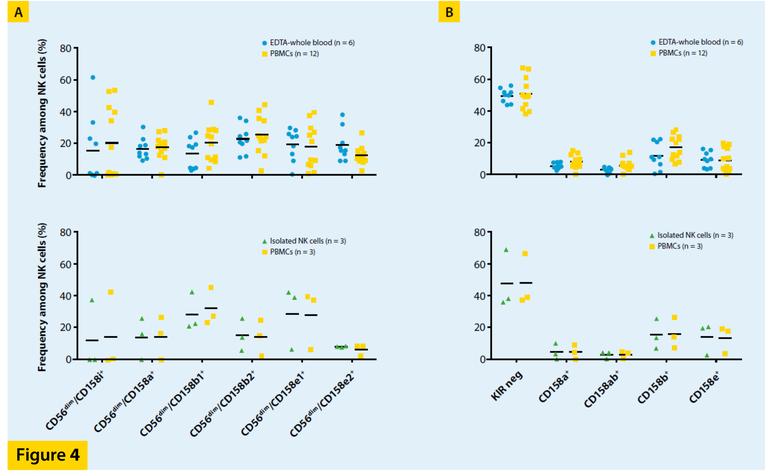


Figure 4

Conclusion

- We established four panels of fluorochrome-conjugated antibodies allowing the flow cytometric analysis of i) 24 NK cell receptors on NK cells and their subsets derived from EDTA–whole blood or PBMCs, and ii) 19 NK cell receptors on isolated NK cells.
- It was possible to compensate the spectral overlap retrospectively using single-stained beads and the FlowLogic Software, which reduced hands-on time.
- These analytical tools have great potential to facilitate donor selection before HSCT and monitoring of NK cell receptors after HSCT with haploidentical TCRα/β⁺ T cell– and B cell–depleted grafts, for example.

References

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3. Cooley, S. *et al.* (2010) Blood 116: 2411–2419.
4. Pfeiffer, M.M. *et al.* (2010) Haematologica 95: 1381–1388.

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