Standardized and flexible eight-color flow cytometry panels harmonized between different laboratories to study human NK cell phenotype and function

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Introduction

Advancements in flow cytometry led to the development of multicolor antibody panels, which have found application in numerous fields. However, comparability of data obtained from different laboratories is still a major challenge. Harmonization of flow cytometry panels and protocols for data analysis will be essential to compare data from various centers. Therefore, we developed standardized flow cytometry panels and independently established acquisition protocols for three different flow cytometers (BD™ FACSCanto™ II, BD LSRFortessa™, and MACSQuant® Analyzer 10), with compatible lasers and filter settings. Eight-color flow cytometry protocols were designed to study natural killer (NK) cell receptor phenotype and function (e.g. antibody dependent cell mediated cytotoxicity (ADCC)) of NK cells within peripheral blood mononuclear cells (PBMCs). Using our established SOPs and harmonized flow cytometry panels, we compared the effects of freezing and thawing of PBMCs on NK cell phenotype and function.

Materials and methods

1 Instruments and reagents

Detectors	Filter settings: band pass and long pass (LP) filters			PMT voltages		
	BD FACSCanto II	BD LSRFortessa	MACSQuant Analyzer 10	BD FACSCanto II	BD LSRFortessa	MACSQuant Analyzer 10
FSC	488/10	488/10	488/10	319	489	328
SSC	488 /10	488/10	488/10	462	266	476
FL1	530/30 and 502LP	530/30 and 505LP	525/50	496	484	417
FL2	585/42 and 556LP	575/26 and 550LP	585/40	435	473	416
FL3	780/60 and 735LP	780/60 and 750LP	750LP	540	549	487
FL4	670LP and 655LP	695/40 and 685LP	655–730	507	681	594
FL5	660/20	670/14 and 655LP	655–730	588	484	522
FL6	780/60 and 735LP	780/60 and 750LP	750LP	474	451	579
FL7	450/50	450/50	450/50	375	423	444
FL8	510/50 and 502LP	525/50 and 505LP	525/50	403	453	560

Table 1 Instrument settings of the three different flow cytometers used in this study.

Panel	Laser	Antibody	Fluorochrome	Clone	Titration	Manufacturer	Order no.
NK phenotype panel	Violet 405 nm	CD45	VioGreen™	5B1	1:11	Miltenyi Biotec	130-096-906
		CD3	VioBlue®	BW264/56	1:11	Miltenyi Biotec	130-094-363
		TCRγ/δ	VioBlue	11F2	1:11	Miltenyi Biotec	130-101-557
		CD14	VioBlue	TÜK4	1:11	Miltenyi Biotec	130-094-364
		CD19	VioBlue	LT19	1:11	Miltenyi Biotec	130-098-598
		SYTOX® Blue	Dead cell marker		1:1000	Thermo Fisher Scientific	S11348
	Blue 488 nm	KIR2D	FITC	NKVFS1	1:11	Miltenyi Biotec	130-098-689
		NKG2A	PE-Vio® 770	REA110	1:11	Miltenyi Biotec	130-105-647
		NKG2C	PE	REA205	1:11	Miltenyi Biotec	130-103-635
		NKG2D	PerCP-Cy™5.5	1D11	1:11	BioLegend	320818
	Red 633 nm	CD16	APC	VEP13	1:11	Miltenyi Biotec	130-091-246
		CD56	APC-Vio 770	REA196	1:11	Miltenyi Biotec	130-100-694
NK function panel	Violet 405 nm	CD45	VioGreen	5B1	1:11	Miltenyi Biotec	130-096-906
		CD14	VioBlue	TÜK4	1:11	Miltenyi Biotec	130-094-364
		CD19	VioBlue	LT19	1:11	Miltenyi Biotec	130-098-598
		SYTOX® Blue	Dead cell marker		1:1000	Thermo Fisher Scientific	S11348
	Blue 488 nm	CD25	VioBright™ FITC	4E3	1:11	Miltenyi Biotec	130-104-274
		CD107a	PE	H4A3	1:11	Miltenyi Biotec	130-095-515
		NKp44	PE-Vio 770	2.29	1:11	Miltenyi Biotec	130-104-195
		CD3	PerCP-Vio 700	BW264/56	1:11	Miltenyi Biotec	130-097-582
		TCRγ/δ	PerCP-Vio 700	11F2	1:11	Miltenyi Biotec	130-103-784
	Red 633 nm	CD16	APC	VEP13	1:11	Miltenyi Biotec	130-091-246
		CD56	APC-Vio 770	REA196	1:11	Miltenyi Biotec	130-100-694

Table 2 Antibodies detected based on the violet and red lasers (indicated in dark blue) represent the panel backbone, antibodies detected based on the blue laser (indicated in light blue) represent the drop-in antigens.

Results

1 Uniform gating strategy to generate reproducible data between flow cytometers

A solid backbone concept enabled exclusion of non-NK lymphocyte populations and dead cells, followed by gating on viable NK cells (CD45⁺CD3⁻CD56⁺ cells). Flexible addition of further antibodies allowed the detailed analysis of NK cell receptors CD16, NKG2A, NKG2C, NKG2D, and KIR2D (A) and NK cell functionality markers CD25, NKp44, and CD107a (B).

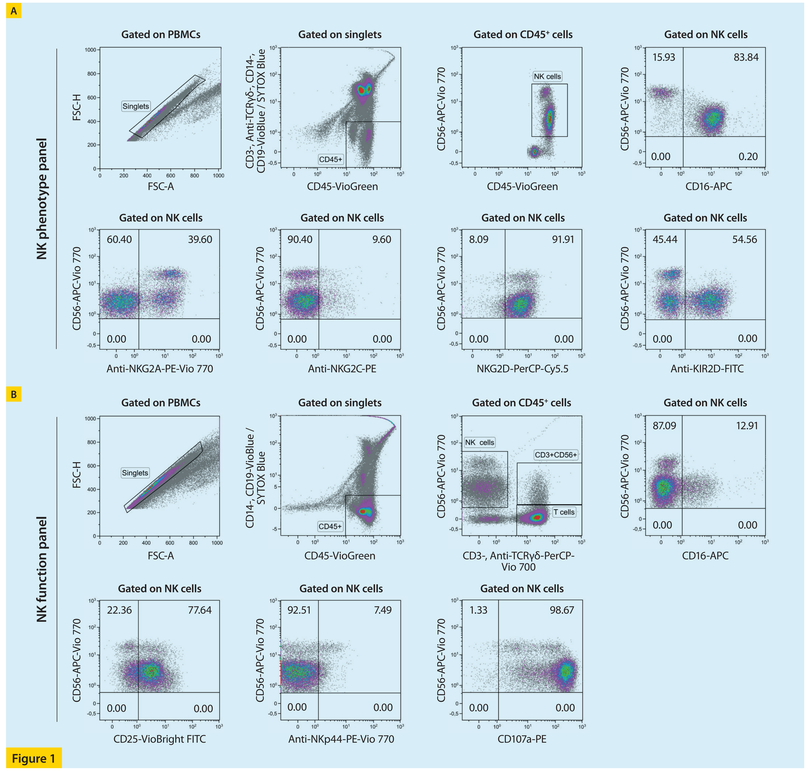

Figure 1

2 Fluorescence intensities of defined fluorochrome-conjugated antibodies are comparable between three flow cytometers

Compatible instrument settings and optimized protocols resulted in comparable data sets as reflected by expression levels on single-stained cells for each backbone and drop-in antigen of the two panels. One representative set of histograms from three samples obtained from a single healthy donor is shown for each antibody-fluorochrome conjugate tested using three different flow cytometers. Data collected from individual experiments were analyzed using the pre-defined gating strategy with KALUZA® software. No significant differences could be observed between centers, resulting in highly reproducible data sets for the characterization of lymphocyte subsets and NK cell phenotype and function. This result provides a strong rationale for combining data sets for analysis in multicenter studies.

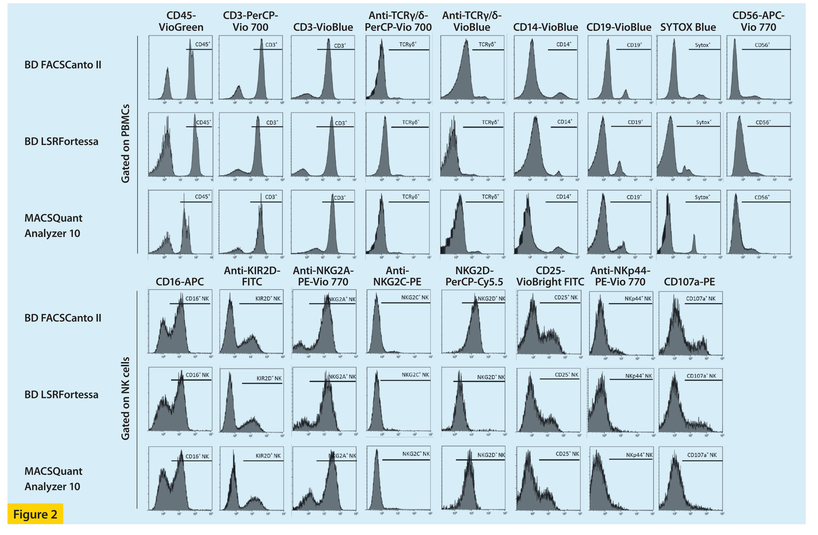

Figure 2

3 NK cell phenotypes and functions are highly preserved after cryopreservation

Additionally, we aimed to identify the most suitable conditions to study NK cell phenotype and function in a multicenter setting. Both freshly isolated and corresponding cryopreserved PBMC samples (n = 12) were either activated with cytokines (IL-2/IL-15) overnight or left untreated. NK cell phenotype and cytotoxicity against A431 cells alone or coated with cetuximab antibody (CET) were similar in fresh and cryopreserved PBMCs, suggesting that the use of cryopreserved PBMCs is appropriate for NK cell studies in a multicenter setting.

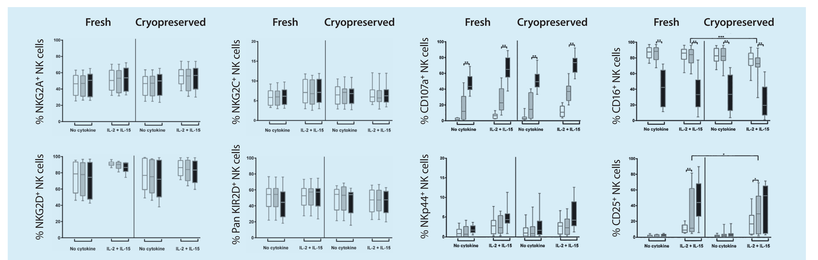

Figure 3

Conclusion and outlook

- Comparable and reproducible data from three different flow cytometers were obtained using optimized 8-color flow cytometry panels for the analysis of NK cells.
- Channels corresponding to drop-in antigens can be flexibly used to obtain novel phenotypic or functional information.
- Cryopreserved PBMCs might be preferably used for studies on NK cell phenotype and function in multicenter trials.
- The presented NK flow cytometry panels set a precedent for future harmonization of multicolor panels focussed on other immune cell types.

These data are part of the following publication:
Veluchamy, J. P. *et al.* (2017) Sci. Rep. 7: 43873.

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