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OMIP-029: Human NK-Cell Phenotypization

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Additional and updated Supporting Information including technical details may be found in the online version of this article.

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Purpose and Appropriate Sample Types

The present panel was optimized to enumerate natural killer (NK) cells within peripheral blood mononuclear cells (PBMC) and to determine their phenotype in terms of NK receptor and differentiation marker expression in healthy individuals. It works well with cryopreserved PBMC and we have observed similar results with fresh specimens. Other tissue types have not been tested (Table 1).

Background

NK-cells are a type of large granular lymphocytes that can generate a fast immune response against infected or transformed cells. Subsets of NK-cells can be differentiated by their relative cell surface expression of the low affinity Fc receptor FcγRIII (CD16) and the neural cell adhesion molecule (NCAM; also known as CD56). While the latter is important for cell–cell adhesion, CD16 binds the Fc domain of IgG antibodies (Abs), thus inducing Ab-dependent cellular cytotoxicity (ADCC). The NK-receptor CD2 is crucial in the formation of nanotubes (1), which facilitate cytotoxic function by adhesion to motile target cells and transportation of lytic granules for long-distance cytolysis (2). Thus, in addition to identifying NK-cells by the expression of CD16 and/or CD56 on $CD3^-$ $CD4^-$ cells, we selected $CD2^+$ events to focus on those cells most likely to be involved in cytotoxicity (Fig. 1A).

The activation of NK-cells is determined by a balancing act between activating and inhibitory receptors. NKG2D (natural killer Group 2, member D; also known as CD314), NKp30 (CD337), NKp44 (CD336), and NKp46 (CD335) are natural cytotoxicity receptors (NCRs). We did not include NKp44 in the panel, as it is largely absent in freshly isolated PBMC (3). NKp46 is the prototype NCR and expressed on both resting and activated NK-cells; its density correlates with the cells' natural cytotoxicity (4). The cellular expression pattern of NKp30 is similar to that of NKp46, and this molecule is responsible for NKp46-independent lysis (5). Lastly, the activating C-type lectin NKG2D binds a variety of stress ligands, such as the stress-inducible major histocompatibility (MHC) class I chain-related proteins MICA and MICB, which are widely expressed on tumor cells (6) and virally infected cells, as well as UL16-binding proteins (7).

In order to prevent the inadvertent killing of autologous cells, NK-cells express a wide array of killer immunoglobulin-like receptors (KIRs) that inhibit activation and lytic activity upon engagement of autologous MHC class I molecules. The number and type of inherited KIR genes differs between individuals and is largely linked to their MHC haplotypes (8). In addition to the NCRs described above, the present panel addresses the expression of the inhibitory receptors, as well as of the homing receptors C-C chemokine receptor type 7 (CCR7; also known as CD197) and L-selectin (CD62L) (Fig. 1B). Since KIRs are both polymorphic and highly homologous, the two KIR Abs used in this panel each react with more than one receptor: clone HP-MA4 recognizes KIR2DL1 (CD158a), KIR2DS1 (CD158h), KIR2DS3, and KIR2DS5 (CD158g), while clone DX27 binds to KIR2DL2 (CD158b1), KIR2DL3 (CD158b2), and KIR2DS2 (CD158j). Thus, staining does not indicate the expression of a single

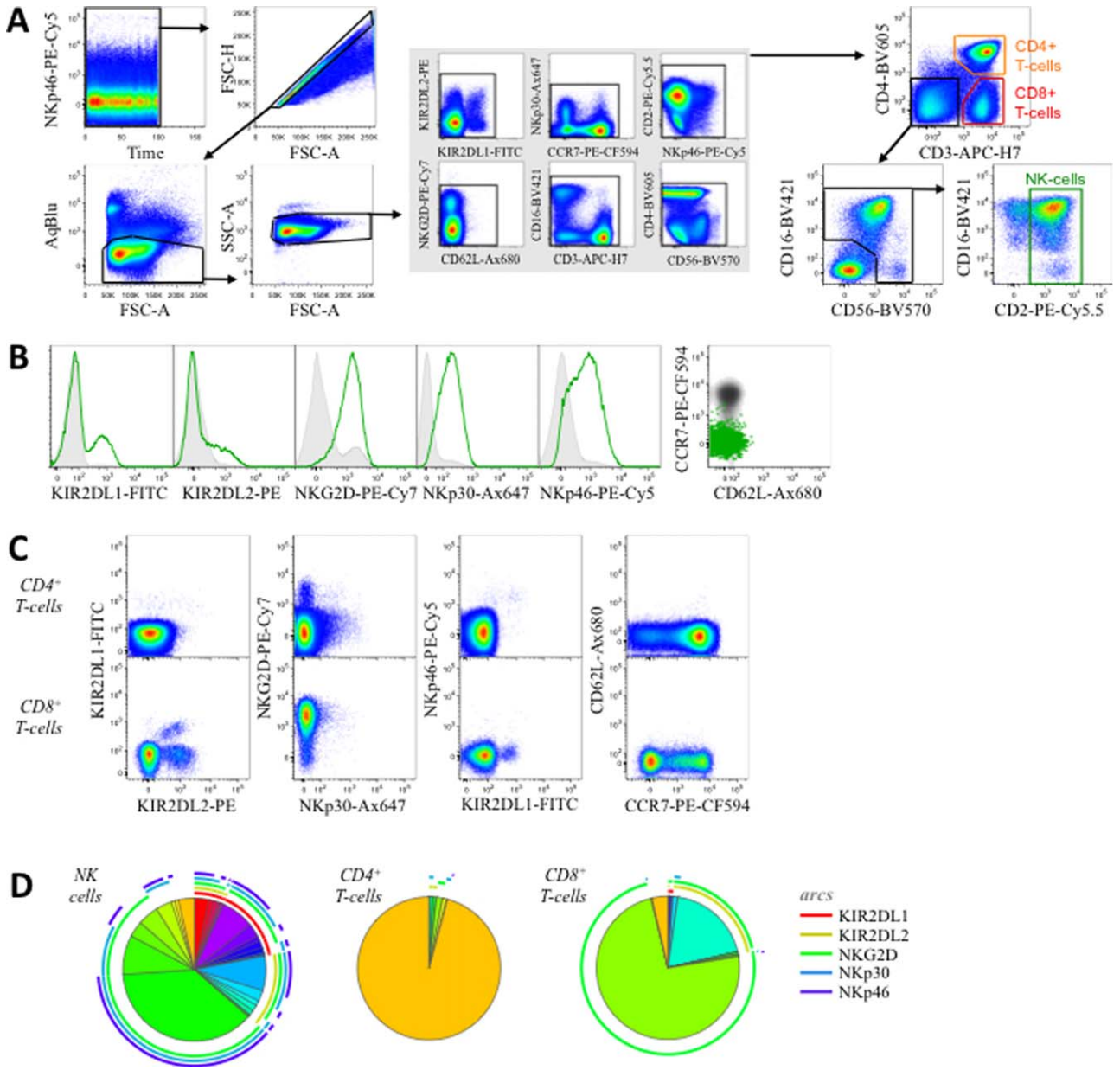

Figure 1. Example staining and gating. **A**: Identification of NK-cells as well as of $CD4^+$ and $CD8^+$ T-cells. After verifying that there were no irregularities in the acquisition over time ($NKp46^{PE\text{-}Cy5}$ vs. Time), single cells were selected (FSC-H vs. FSC-A) before gating on live ($AqBlu^-$) small lymphocytes (SSC-A vs. FSC-A). Any reagent aggregates were gated out (gray box). After excluding any cells expressing either CD3 or CD4, $CD2^+$ NK-cells (green gate) were identified within the subset expressing CD16 and/or CD56. Within non-NK-cells, $CD4^+$ (orange gate) and $CD8^+$ T-cells (red gate) were also selected for subsequent phenotypic analysis. **B**: Phenotype of NK-cells. The expression of the NK receptors KIR2DL1, KIR2DL2, NKG2D, NKp30, and NKp46, as well as of the differentiation markers CCR7 and CD62L were investigated. NK-cells (green) are overlaid onto total lymphocytes (gray). **C**: Expression of NK receptors and differentiation markers on T-cells. Cell surface expression of the same molecules described in (B) were analyzed on both $CD4^+$ and $CD8^+$ T-cells. **D**: Co-expression pattern of NK receptors. The co-expression pattern of the NK receptors KIR2DL1, KIR2DL2, NKG2D, NKp30, and NKp46 on the surface of NK-cells, $CD4^+$ T-cells, and $CD8^+$ T-cells was investigated. Individual pie slices represent the fraction of cells expressing a given combination of these five markers as shown by the arcs.

Table 1. Summary table for application of OMIP-029

PURPOSE	NK-CELLS
Species	Human
Cell types	PBMC
Cross-references	n.a.

type of receptor, but of a group of receptors. They will here be referred to as KIR2DL1 and KIR2DL2 for simplicity's sake.

Both activating (NCRs) and inhibitory (KIRs) NK-receptors can be expressed by T-cells where they act as co-stimulatory or inhibitory molecules to fine-tune immune responses. Since CD3 and CD4 were used as exclusion markers

Table 2. Reagents used for OMIP-029

SPECIFICITY	ALTERNATIVE NAME	CLONE	FLUOROCHROME	PURPOSE
CD2	LFA-2	S5.5	PE-Cy5.5	NK cells
CD16	FcγRIII	3G8	BV421	
CD56	NCAM	HCD56	BV570	
CD3		SK7	APC-H7	non-NK cells
CD4		OKT4	BV605	
CD158a	KIR2DL1	HP-MA4	FITC	NK receptors
CD158b	KIR2DL2	DX27	PE	
CD314	NKG2D	1D11	PE-Cy7	
CD335	NKp46	BAB281	PE-Cy5	
CD337	NKp30	P30-15	Ax647	
CD62L	L-selectin	SK11	Ax680	Differentiation
CD197	CCR7	150503	PE-CF594	
Dead cells		–	AqBlu	Dump

PE, R-phycoerythrin; Cy, cyanine; BV, brilliant violet; APC, allophycocyanin; H7, highlight 750; FITC, fluorescein; Ax, Alexa; CF, cyanine-based fluorescent dye; AqBlu, LIVE/DEAD Fixable Aqua Dead Cell Stain.

to select a pure NK-cell population, we used these markers to also interrogate the expression of NK-receptors on the surface of $CD4^+$ and $CD8^+$ (gated as $CD4^-$) T-cells (Fig. 1C). By creating Boolean gates, the co-expression pattern of the different receptors can then be investigated on NK-cells, as well as on $CD4^+$ and $CD8^+$ T-cells. Representing such co-expression patterns in pie charts, each pie slice stands for a different combination of NCRs and KIRs, as indicated by the adjacent arcs (Fig. 1D).

All reagents were titrated and compared with other conjugates for performance before investigating their compatibility with reagents to other cell surface markers (see Supporting Information Material). Add-in experiments were performed in order to evaluate potential effects on the detection of unrelated markers as previously described (9). In view of developing a panel with minimal background staining and spillover between detectors, different reagent combinations were tested. The reagents included in the final panel are listed in Table 2.

Similarity to Published OMIPs

In OMIP-007, Eller and Currier published a panel for the phenotypic analysis of human NK-cells (10), which, unlike the present panel, does not include NCRs.

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