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OMIP-115: High-Dimensional Phenotypic Characterization of Human Natural Killer Cells for Therapeutic Use

Emil Birch Christensen | Moritz Schaefer | Mike Bogetofte Barnkob | Christian Nielsen | Torben Barington

Centre for Cellular Immunotherapy of Haematological Cancer Odense (CITCO), Department of Clinical Immunology, Odense University Hospital, University of Southern Denmark, Odense, Denmark

Correspondence: Torben Barington (torben.barington@rsyd.dk)

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ABSTRACT

This 29-color flow cytometry panel was developed and optimized for in-depth characterization of human peripheral blood NK cells for preclinical development and monitoring of NK cell therapies. The panel includes markers associated with NK cell differentiation, cytotoxicity, tissue residency, as well as NK cell dysfunction. Panel optimization was performed on freshly isolated and ex vivo activated NK cells enriched from human peripheral blood mononuclear cells (PBMCs). Overall, this panel functions as a tool to extensively characterize human NK cells, paving the way for rapid and standardized approaches to evaluate the biological activity of therapeutic NK cell products.

1 | Purpose and Appropriate Sample Type

We developed a 29-color panel for extensive phenotypic characterization of NK cells from human peripheral blood to characterize NK cell memory phenotype and activation profile during and after expansion for testing in preclinical models and for monitoring of NK cell therapies. The panel allows for examination of activating and inhibitory receptors that regulate the cytotoxic potential of NK cells, as well as differentiation and adhesion molecules that regulate the migration behavior and functional potential of NK cells (Table 1).

2 | Background

Natural Killer (NK) cells are large granular lymphocytes and represent 5%–20% of peripheral blood mononuclear cells in humans [1]. NK cells play an important role in the innate immune response against viral infections and the elimination of stressed and transformed cells. Unlike T and B cells that recognize antigens through a single somatically rearranged receptor, NK cells recognize target cells through an array of germline-encoded activating and inhibitory receptors [reviewed in [2]]. It is the integration and net balance of signals received from these receptors that activate or inhibit effector functions and enable NK cells to discriminate healthy cells from infected or transformed cells. Besides their direct cytotoxic functions, NK cells regulate immune responses by secreting pro-inflammatory cytokines, such as interferon gamma (IFN-γ) and tumor necrosis factor (TNF).

NK cells have gained significant attention in clinical applications, especially in cancer immunotherapy, due to the intrinsic ability of NK cells to spontaneously eliminate transformed cells and their lack of alloreactivity, thus enabling "off-the-shelf" cellular therapies using this cell type [3]. Additionally, NK cells can be genetically engineered to express a chimeric antigen receptor (CAR) recognizing tumor- or cell lineage-associated antigens, thereby providing enhanced specificity of the NK cells toward the malignant cells [4]. However,

TABLE 1 | Summary table for application of OMIP-115.

Purpose	Comprehensive immunophenotyping of human NK cells
Species	Human
Cell type	PBMCs
Cross-reference	OMIP-007, OMIP-027, OMIP-029, OMIP-039, OMIP-058, OMIP-064, OMIP-070, OMIP-080, OMIP-098

preparation of cell-based therapies differs greatly from conventional small-molecule drugs, and the potency of the final product can vary greatly depending on the protocol used to prepare the cell product. Lack of standardized protocols used to manufacture and evaluate the potency of cell therapy products, as well as inter-donor variability, can result in variations in batch-to-batch quality and presumably clinical outcomes. In addition, NK cells obtained from peripheral blood are highly heterogeneous when it comes to their phenotype and biological activity, which further complicates which criteria are needed for assessing the quality of the final product [5, 6]. Hence, there is a need for improved tools to evaluate the characteristics of NK cell-based products and to define biomarkers that function as potential predictors of in vivo biological activity and potency.

Here, we have developed a 29-color flow cytometry panel for in-depth phenotypic characterization of human peripheral blood NK cells for therapeutic use (Table 2). We tested the panel on freshly isolated and ex vivo expanded NK cells from human PBMCs from healthy donors. Detailed descriptions of the panel design and optimization process are provided in Appendix S1 and Supplementary Figures 1–5.

NK cells can be defined by their surface expression of CD56 and/or CD16, and absence of CD3. We used a dump channel including CD3, CD14, CD19, and a live/dead dye to exclude T cells, monocytes, B cells, and dead cells, respectively (Figure 1A). We additionally included 24 surface markers relevant to assessing NK cell differentiation, cytotoxicity, tissue residency, and dysfunction.

The biological activity of NK cells is partly dependent on their level of differentiation. Immature $CD56^{bright}$ NK cell subsets produce more cytokines and proliferate more, whereas mature $CD56^{dim}$ NK cells are more cytotoxic [7]. Maturation of NK cells is generally characterized by a gradual loss of CD159a (NKG2A), accompanied by the acquisition of CD57 and killer cell immunoglobulin-like receptor (KIR) surface expression [8, 9]. CD159a is a strong inhibitory receptor on NK cells that senses the overall expression level of HLA class I molecules on nearby cells, and it is important for the regulation of self/non-self discrimination [10]. Hence, we included CD159a, CD57, and KIRs in the panel to enable determination of the differentiated state of NK cell populations (Figure 1B). We also included the activating receptor CD159c (NKG2C) to identify a highly functional subset of mature NK cells that displays features of adaptive immunity. CD159c expressing NK cells constitute a distinct subset of NK cells that undergo clonal expansion in response to certain viral infections, such as cytomegalovirus (CMV) infection. CD159c+ NK cells respond more rapidly to viral antigen re-stimulation and form long-lived memory cells [11]. Such features could be attractive in clinical settings because NK cells typically are short-lived, and the presence of CD159c+ memory NK cells may enhance the longevity of NK cell products [12, 13].

NK cells express a range of activating and co-activating receptors that facilitate their cytotoxic responsiveness. To evaluate the cytotoxic profile of NK cells, we included the natural cytotoxicity receptors (NCRs) CD355 (NKp46) and CD337 (NKp30), as well as the activating receptors CD314 (NKG2D) and CD226 (DNAM-1) (Figure 1E). These receptors play important roles in triggering NK cell effector functions by recognizing a variety of stress-induced antigens on infected or transformed cells and transmitting activating signals [reviewed in [14]]. The expression of these receptors has been linked to NK cell subsets with enhanced cytotoxicity and pro-inflammatory properties and thus could potentially function as positive predictors of cytotoxic potential and potency of NK cell products [15]. We also included CD8, which is expressed on a subset of NK cells with enhanced cytotoxicity against multiple tumor target cell lines [16, 17]. In addition to the release of lytic granules, NK cells also mediate the killing of target cells through their interaction with death receptors. We included the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) CD253, which is expressed on activated NK cells and is important for NK cell mediated clearance of stressed and malignant cells [18, 19].

NK cells also express a range of adhesion molecules that participate in the formation of immunological synapses. We included CD11a, which is part of the LFA-1 complex, and CD2 (LFA-2), as they function as important target cell adhesion molecules and provide co-stimulatory molecules for NK cell activation [20–22]. We also included the inhibitory receptors CD328 (Siglec-7) and CD161 (KLRB1) (Figure 1D). Despite negatively regulating NK cell cytotoxicity, both CD328 and CD161 expression has been linked to distinct subsets of highly functional NK cells [23, 24].

NK cells express a range of cytokine and chemokine receptors that dictate their responsiveness to stimulation and tissue residency. Evaluating the migratory potential of NK cell products provides important information about the ability of the NK cells to traffic to and persist in specific tissues, which ultimately affects the potency of the cell therapy product. We included CD195 (CCR5), CD197 (CCR7), CD62L, and CD69 in our panel to assess the migratory potential of NK cells (Figure 1C). The chemokine receptor CD195 functions as a homing marker in the migration of immune cells toward infected tissues and tumors [25]. Expression of CD197 and CD62L both drives the migration of immune cells toward secondary lymphoid tissues but also defines functionally distinct subsets of NK cells. CD197 is predominantly expressed on $CD56^{bright}$ NK cells but can be acquired by cytotoxic $CD56^{dim}$ NK cells from malignant cells through trogocytosis [26, 27]. CD62L-expressing NK cells constitute a subset of polyfunctional NK cells with improved cytotoxicity, cytokine production, and proliferation capacity in vivo [28]. CD69 is used as an early activation marker in lymphocytes and prevents

TABLE 2 | Reagents used in OMIP-115.

Specificity	Alternative name	Clone	Fluorochrome	Purpose
CD2	LFA-2	RPA-2.10	APC/Cyanine7	Adhesion/co-stimulation
CD3	—	UCHT1	V450	Exclusion
CD8	—	SK1	BUV737	Activating
CD11a	LFA-1	HI111	BUV496	Adhesion/co-stimulation
CD14	—	MφP9	V450	Exclusion
CD16	FcγRIII	3G8	BV785	Maturation/activating
CD19	—	HIB19	V450	Exclusion
CD25	IL2RA	M-A251	PE/Fire 700	Activation marker/residency
CD38	ADPRC1	HIT2	BV480	Activation marker
CD56	NCAM	NCAM16.2	RB780	Maturation
CD57	LEU7	HNK-1	PerCP-Cy5.5	Maturation
CD62L	L-selectin	DREG-56	BV750	Residency
CD69	AIM	FN50	APC-R700	Activation/residency
CD85j	ILT2	GHI/75	RB744	Inhibitory
CD158a/h/g	KIR2DL1/S1/S3/S5	HP-MA4	RB744	Activating/inhibitory
CD158b1/b2/j	KIR2DL3/DS2/DL2	DX27	RB744	Activating/inhibitory
CD158e1	KIR3DL1	DX9	RB744	Inhibitory
CD159a	NKG2A	S19004C	APC	Inhibitory/maturation
CD159c	NKG2C	134591	BV711	Maturation/activating
CD161	KLRB1	HP-3G10	BUV661	Activating
CD195	CCR5	2D7/CCR5	BUV615	Residency
CD197	CCR7	G043H7	PE/Dazzle 594	Residency
CD223	LAG-3	11C3C65	BV605	Inhibitory/dysfunction
CD226	DNAM-1	11A8	BV650	Activating
CD253	TRAIL	RIK-2	BUV395	Activating
CD279	PD-1	PD1.3.1.3	PE-Vio770	Inhibitory/dysfunction
CD314	NKG2D	1D11	BB515	Activating
CD328	Siglec-7	6-434	PE	Inhibitory
CD335	NKp46	900	BV421	Activating
CD337	NKp30	p30-15	BUV563	Activating
CD366	TIM-3	F38-2E2	PE/Cyanine5	Inhibitory/dysfunction
KLRG1	—	Z7-205.rMAb	RB613	Inhibitory/dysfunction
TIGIT	—	A15153G	PE/Fire 810	Inhibitory/dysfunction
Viability	—	—	Zombie NIR	Exclusion

egress of tissue resident lymphocytes into peripheral blood [29]. We additionally included NK cell activation markers CD38 and CD25 [30, 31].

NK cells also express several inhibitory and checkpoint receptors that negatively regulate NK cell effector functions. We included CD279 (PD-1), CD366 (TIM-3), CD223 (LAG-3), TIGIT, and KLRG1 to evaluate overall dysfunctional states in NK cells (Figure 1D). Although a lack of consensus exists around the classification of dysfunctional states in NK cells, each of these receptors has been linked to suppressed NK cell functions and could thus function as negative predictors

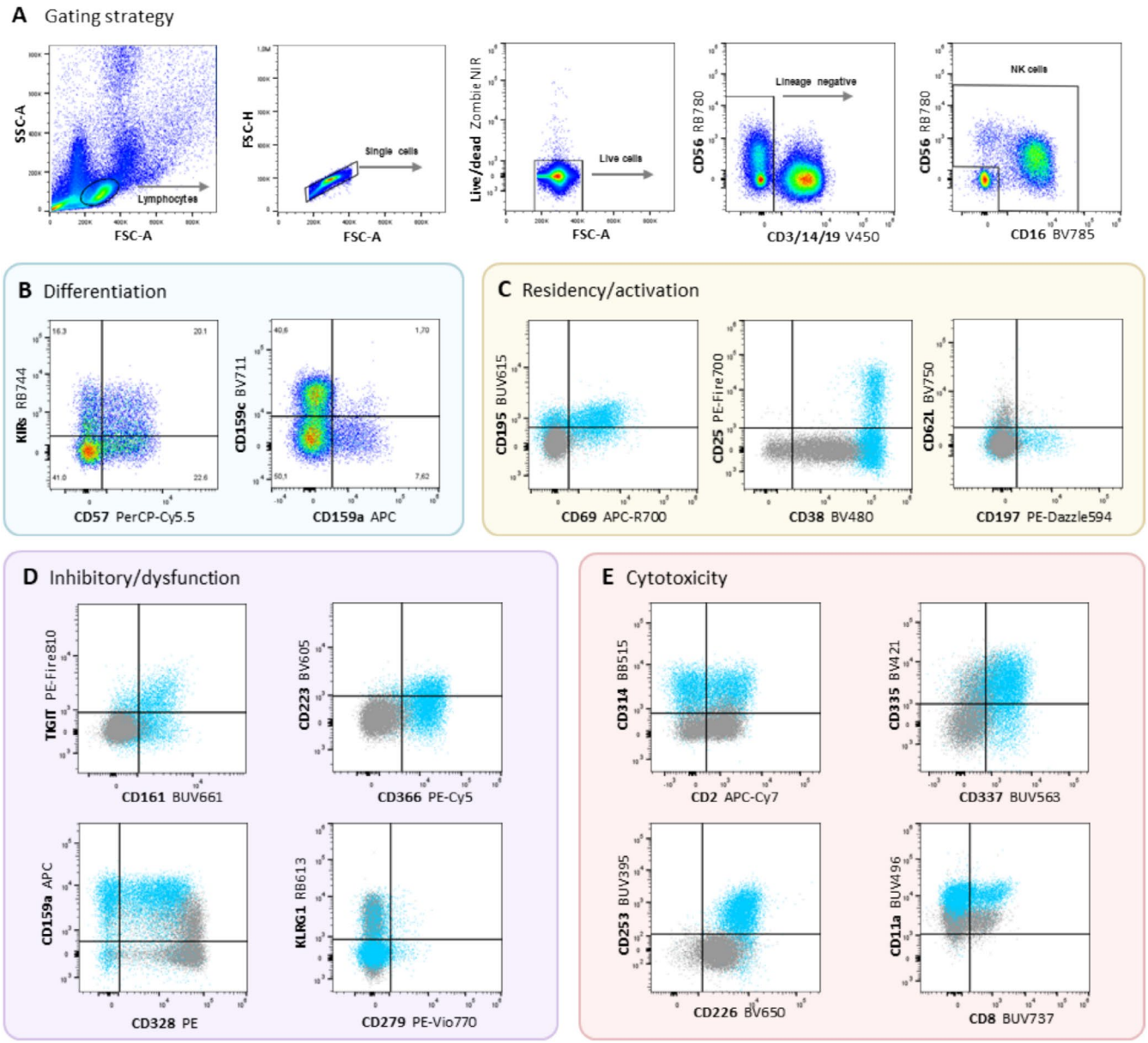

FIGURE 1 | (A) Gating strategy used to identify NK cells from starting PBMC sample. (B) Assessment of developmental diversity of NK cells, based on expression of maturation markers. (C, D) Representative bivariate plots illustrating panel performance on freshly isolated PBMCs (gray dots) and ex vivo cultured NK cells (blue dots). (C) Assessment of activation level and potential tissue residency properties of NK cells. (D) Assessment of dysfunctional states of NK cells, based on the expression of checkpoint and inhibitory receptors. (E) Assessment of cytotoxic potential of NK cells based on the expression of activating and co-activating receptors.

of NK cell effector functions and potency [32–36], especially in cases with co-expression of several of these receptors, as known from T cells [37].

In summary, we have developed a comprehensive flow cytometry panel for in-depth phenotyping of human NK cells. This panel enables a novel and rapid approach to evaluate the characteristics and potency of NK cell-based products, which may ultimately lead to improved treatment regimens and therapeutic outcomes in patients. With its broad range of markers, the panel is suited for additional applications including NK cell characterization during preclinical development of NK products, as well as a diagnostic tool in NK cells obtained from cancer patients.

Author Contributions

Emil Birch Christensen: conceptualization (equal); data curation (lead); formal analysis; funding acquisition (equal); investigation (lead); methodology (lead); project administration; resources (lead); software; validation (lead); visualization (lead); writing – original draft. **Moritz Schaefer:** formal analysis (supporting); investigation (supporting); methodology (supporting); writing – review and editing (equal). **Mike Bogetofte Barnkob:** conceptualization (equal); methodology (equal); supervision (equal); writing – review and editing (equal). **Christian Nielsen:** conceptualization (equal); investigation (supporting); validation (supporting); visualization (supporting); writing – review and editing (equal). **Torben Barington:** conceptualization (equal); funding

acquisition (equal); resources (equal); supervision (lead); writing – review and editing (lead).

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Disclosure

Several published OMIPs including OMIP-007, OMIP-029, OMIP-039, OMIP-058, and OMIP-064 include markers for phenotypic analysis of human NK cells, focusing primarily on broadly defining the major human leukocyte subsets. OMIP-027, OMIP-39, OMIP-070, OMIP-80, and OMIP-098 explore characterization of NK cells and mainly focus on other applications such as functional profiling, tumor tissue diagnostics, and memory NK cell profiling. Our panel encompasses a broad range of NK cell markers involved in several aspects of NK cell functions, enabling in-depth characterization of NK cell products for therapeutic use.

Ethics Statement

Only discarded and fully anonymized blood samples (buffy coats) from routine blood donations were used for purification of NK cells, and this use does not require permission from an ethical committee according to Danish Law. The donors gave written consent to this kind of use at each blood donation.

Conflicts of Interest

M.B.B. has received consulting honorariums from Roche and Kite/Gilead, unrelated to the present work. The remaining authors declare no competing interests.

Data Availability Statement

The data that support the findings of this study are openly available in NK cell phenotyping at http://flowrepository.org/id/FR-FCM-Z79A, reference number FR-FCM-Z79A.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.