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OMIP-007: Phenotypic Analysis of Human Natural Killer Cells

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Additional Supporting Information may be found in the online version of this article.

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• **Key terms**
NK cells; KIR; HIV-1; human

Purpose and Appropriate Sample Types

This panel was developed to characterize the phenotype of human natural killer (NK) cells from cryopreserved peripheral blood mononuclear cells (PBMC) isolated from ACD or EDTA anticoagulated whole blood or apheresis units (Table 1). The application of this panel was to identify changes in NK cell subsets with regard to receptor expression, maturation, homing potential, and activation in the setting of primary HIV-1 natural infection. However, this panel may be applied to a wide variety of disease and normal conditions to characterize NK cells in humans. The performance of this panel was tested on fresh and frozen PBMC as well as using a whole blood lyse no wash procedure.

Background

NK cells are innate effector cells representing ~10% of circulating lymphocytes with more than 2 billion in circulation at any given time (1). NK cells are classically defined by the expression of two cellular markers; CD56, the neural cell adhesion molecule (NCAM) and CD16, the Fcγ-receptor IIIa. These markers (CD56/CD16) allow the discrimination of at least three distinct NK cell subsets and their distribution is altered in HIV-1 infection (2). In developing this panel, commonly used conjugates, CD56 PE and CD16 FITC, were replaced with CD56 PE-Cy7 and CD16 Pac Blue, conjugates that better separated the NK cell subsets as well as to allow for commercial reagents to more exotic receptors in the PE and FITC channel (Table 2). CD8, which performs well with any fluorochrome was included using the tandem dye APC-H7 to further define NK subsets. NK cells are noted for the combination of activating and inhibitory receptors that regulate their activity. Most relevant in the HIV-1 research field are the killer cell immunoglobulin-like receptors (KIRs) as a number of genetic associations are observed between certain KIR and HLA combinations with regard to HIV-1 disease progression [reviewed by Bashirova et al. (3)]. A very limited selection of antibodies directly conjugated to FITC, APC, and PE, are available for a subset of the KIR receptors with highly variable staining patterns typically observed. The KIR receptors that bind HLA-A, HLA-B, and HLA-C were most interesting and all combinations were procured and tested to identify the best signal-to-noise ratio. The KIR3DL1 demonstrates clear separation of positive cell populations in FITC, PE, and APC but we selected the Alexa Fluor® 700 conjugate since this conjugate shows sufficient separation between positive and negative populations and is in a less common channel. Similarly, the KIR2DL1/DS1 (clone HP-MA4) antibody demonstrates clear separation of positive cell populations in FITC and PE but we chose the tandem conjugate PerCP-Cy5.5 because of the highest signal-to-noise ratio. The KIR2DL3/DL3/DS2 antibody is available in FITC, PE, and APC but showed the greatest separation in PE. Based on limited reagent availability and the design of a more generalizable panel, we did not include KIR receptor reagents to discriminate activating forms of these receptors.

KIR expression along with CD57 and NKG2A allow for the discrimination of a proposed NK cell differentiation process (4). CD57 (clone HCD57) is available in Alexa

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Table 1. Summary table for OMIP-007

Purpose	Characterize the phenotype, maturation, and activation of NK cells
Species	Human
Cell types	Fresh and cryopreserved PBMC
Cross references	None

Fluor 647, APC, FITC, Pac Blue, and PE and has clear separation of positive populations in Pac Blue and APC but because CD16 is optimal on Pac Blue we used CD57 APC. We evaluated NKG2A, clone Z199, (Supporting Information Fig. 4H), which allows for easy discrimination of positive cells in PE and APC, but ultimately decided to use only the KIR antibodies in combination with CD57 APC to characterize NK cell differentiation. In addition to maturation, we were interested in studying homing marker expression. Both CCR7 and CD62L have been associated with trafficking out of the peripheral blood and into secondary lymphatic tissue. CCR7 has been reported to be expressed exclusively on CD56bright NK cells (5). We found CCR7 expression on the NK cell CD56 bright population at prohibitively low intensity and excluded this marker from our panel (Supporting Information Fig. 3). L-Selectin (CD62L) is linked to extravasation into tissues and has been associated with increased functional potential in various NK cell subsets (6,7). CD62L (clone DREG56) is commercially available in several fluorochromes and shows clear separation of positive populations in both Alexa Fluor 700 and conjugated to Qdot605, which was selected to be included in this panel. Another homing marker, α4β7 has recently been shown to mark an expanded NK population in SIV infection and may be of interest in primary HIV-1 infection (8). The α4β7 (ACT-1) monoclonal antibody from the NIH AIDS Reference and Reagent program was directly conjugated to eF650 nanocrystals for inclusion in this NK panel. Finally, NK cell activation was marked using

Table 2. Reagents used in OMIP-007

SPECIFICITY	CLONE	FLUOROCHROME	PURPOSE
Live/Dead	NA	Aqua	Viability
CD3	S4.1	PE-TR	Exclusion
CD4	SFCI12T4D11	ECD	
CD14	TuK4	PE-Cy5	
CD19	SJ25-C1	PE-Cy5	
CD16	3G8	PB	NK subsets
CD56	NCAM16.2	PE-Cy7	
CD8	SK1	APC-H7	
α4β7	ACT-1	Qdot 655	Homing
CD62L	DREG56	Qdot 605	
KIR2DL1/DS1	HP-MA4	PerCP-Cy5.5	KIR receptors
KIR2DL2/DS2/DL3	DX27	PE	
KIR3DL1	DX9	Alexa700	
HLA-DR	G46-6	FITC	Activation
CD57	HCD57	APC	Differentiation

NA, not applicable; APC, allophycocyanin; Cy, cyariin; ECD, energy-coupled dye; FITC, fluorescein isothiocyanate; PE, R-phycoerythrin; PerCP, peridinin chlorophyll A protein; PETR, PE Texas Red; QDot, quantum dot.

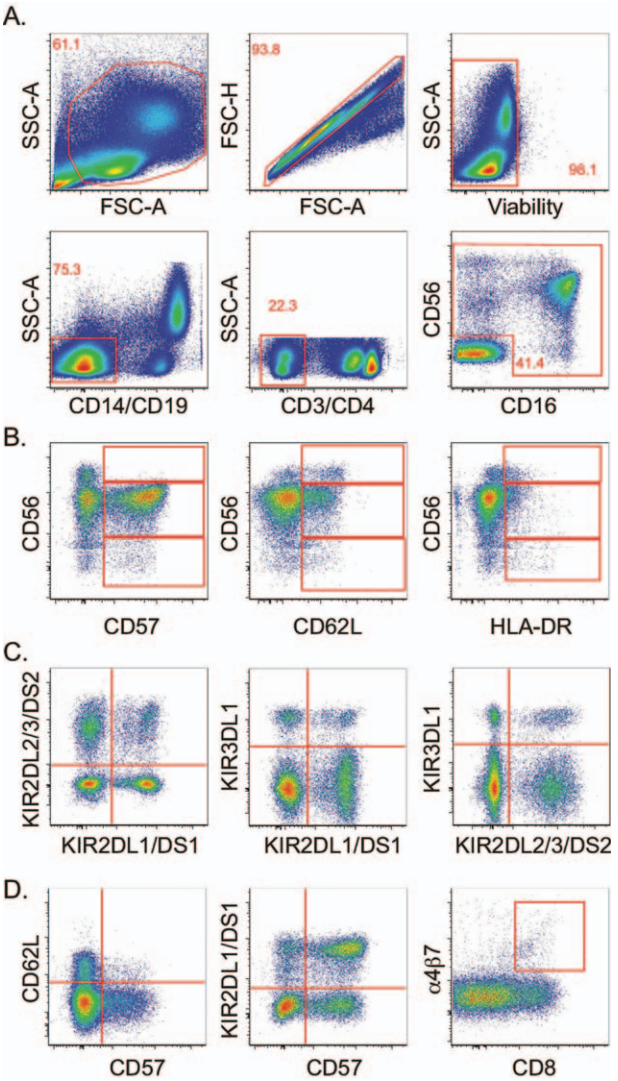

Figure 1. Gating strategy and panel performance for OMIP-007. PBMC were thawed and stained with the human NK cell panel as outlined in the Supporting Information. **A**: Overall, successive gating strategy demonstrates initial broad gating on forward and side scatter to include large lymphocytes. Forward area and height are used to discriminate single cells followed by identification of viable cells using an amine reactive dye. Monocytes and B cells are excluded using CD14 and CD19 and T cells are excluded using CD3 and CD4. It is important to exclude dim CD4 as some DC populations may express this marker. CD56 and CD16 are used to identify NK cells discriminating between multiple populations on the basis of $CD56_{bright}$, $CD56_{dim}$, and $CD56_{negative}$ expression levels. **B**: The relative amount of CD57, CD62L, and HLA-DR expression in the distinct NK subsets with example gating on the $CD56_{bright}$, $CD56_{dim}$, and $CD56_{negative}$ NK cell subsets. **C**: Variable combinations of the three KIR antibodies identified cells possess multiple activating and inhibitory receptors on the overall NK cell compartment, with proposed gating demonstrated. **D**: All three panels are derived from the overall NK cell gate outlined (A). Example analysis of unique aspects of the NK panel showing the mutually exclusive nature of CD62L and CD57 in a normal healthy adult is shown. KIR expression with CD57 distinguished mature populations of NK cells. The homing marker α4β7 is expressed on a subset of NK cells expressing CD8. All gates were defined using FMO and unstained samples to discriminate positive and negative signals.

HLA-DR FITC, although this antibody demonstrates good fluorescent intensity compared with negative populations using V450 and APC. The performance of the NK panel is displayed in Figure 1.

Similarity to Published OMIPs

None to date

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