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OMIP-039: Detection and Analysis of Human Adaptive NKG2C$^+$ Natural Killer Cells

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• **Key terms**
human; NK cells; adaptive NK cells; NK receptors; NKG2C; cytomegalovirus

Purpose and Appropriate Sample Types

THE present panel was optimized for the detection of adaptive natural killer (NK)-cell populations in healthy human donors and offers versatility to investigate their biology including receptor usage, activation requirements, or signaling adaptor and transcription factor expression. It was established on cryopreserved PBMC and yields similar results with freshly isolated PBMC. Additional tissue samples have not been tested (Table 1).

Background

Natural Killer (NK) cells are members of the innate lymphoid cell (ILC) family (1) and provide immediate immune responses against transformed and virally infected cells (2). Although NK cells are classically regarded as part of the innate immune system, accumulating evidence indicates that NK cells can mediate immunological memory and display adaptive features, specifically in response to viral infections (3,4). The contribution of human NK-cell responses to the defense against viral infections is highlighted by rare cases of patients with genetic NK-cell deficiencies, who suffer from severe and disseminated infections, especially caused by herpesviruses such as human cytomegalovirus (HCMV) (5).

In contrast to virus-specific CD8$^+$ or CD4$^+$ T cells, adaptations of the NK-cell compartment to HCMV are not detected in all, but only in 30–40% of HCMV-seropositive healthy individuals (6,7). In these individuals, the activating receptor NKG2C (CD159c) as well as activating killer cell immunoglobulin-like receptors (KIR) mark a subset of NK cells, which has adapted to HCMV (6,8) and has, therefore, previously been termed "adaptive NK cells" (9).

Several peculiar features of adaptive NK cells such as epigenetic alterations (9,10) and functional properties (11) have been defined, while many other aspects remain incompletely understood or unexplored. To further investigate adaptive NK cells and address open questions, it is essential to definitively discriminate between adaptive NKG2C$^+$ NK cells, which were generated by HCMV infection in a sub-group of HCMV-seropositive individuals, and conventional NKG2C$^+$ NK cells, which are present at low frequencies at steady state even in HCMV-seronegative individuals. Since the proportion of NKG2C$^+$ NK cells in HCMV-seropositive donors varies considerably (6,8,11), the frequency of NKG2C-expressing CD56^{dim} NK cells is not a sufficient criterion to identify adaptive NKG2C$^+$ NK cells, although setting thresholds has been previously proposed (12). Consequently, assessing other attributes is required to reliably detect adaptive NK cells in HCMV-seropositive individuals.

Preferential expression of self-HLA class I-binding inhibitory KIR is a hallmark of adaptive NK cells (6,8,11). Hence, identification of adaptive NK-cell subsets as

Table 1. Summary table for OMIP-039

Purpose	Detect adaptive NK cells and serve as a backbone to assess their biological properties
Species	Human
Cell types	Cryopreserved and freshly isolated PBMC
Cross references	OMIP-007, OMIP-027, and OMIP-029

outliers from the stochastic expression pattern of KIR is an elegant approach, which has proven essential to study adaptive NK-cell responses (6). However, this detection strategy requires KIR and HLA genotyping combined with a series of stringent quality control procedures to overcome inherent limitations due to cross-reactive reagents (13).

Another central feature of adaptive NK cells is a skewed receptor repertoire compared to conventional NK cells (reviewed in Ref. 14) and numerous differentially expressed surface receptors have been reported (6,8,11). However, large heterogeneity in the NK-cell compartment among individuals (15) renders the use of single markers to detect adaptive NK cells challenging. To address this issue, the present 11-fluorochrome panel combines seven previously characterized surface markers and allows to detect adaptive NK cells based on a combinatorial expression pattern. The panel was designed to serve as a backbone for implementing additional markers of interest to further characterize the phenotype and functional properties of adaptive NK cells in a broad range of experimental settings.

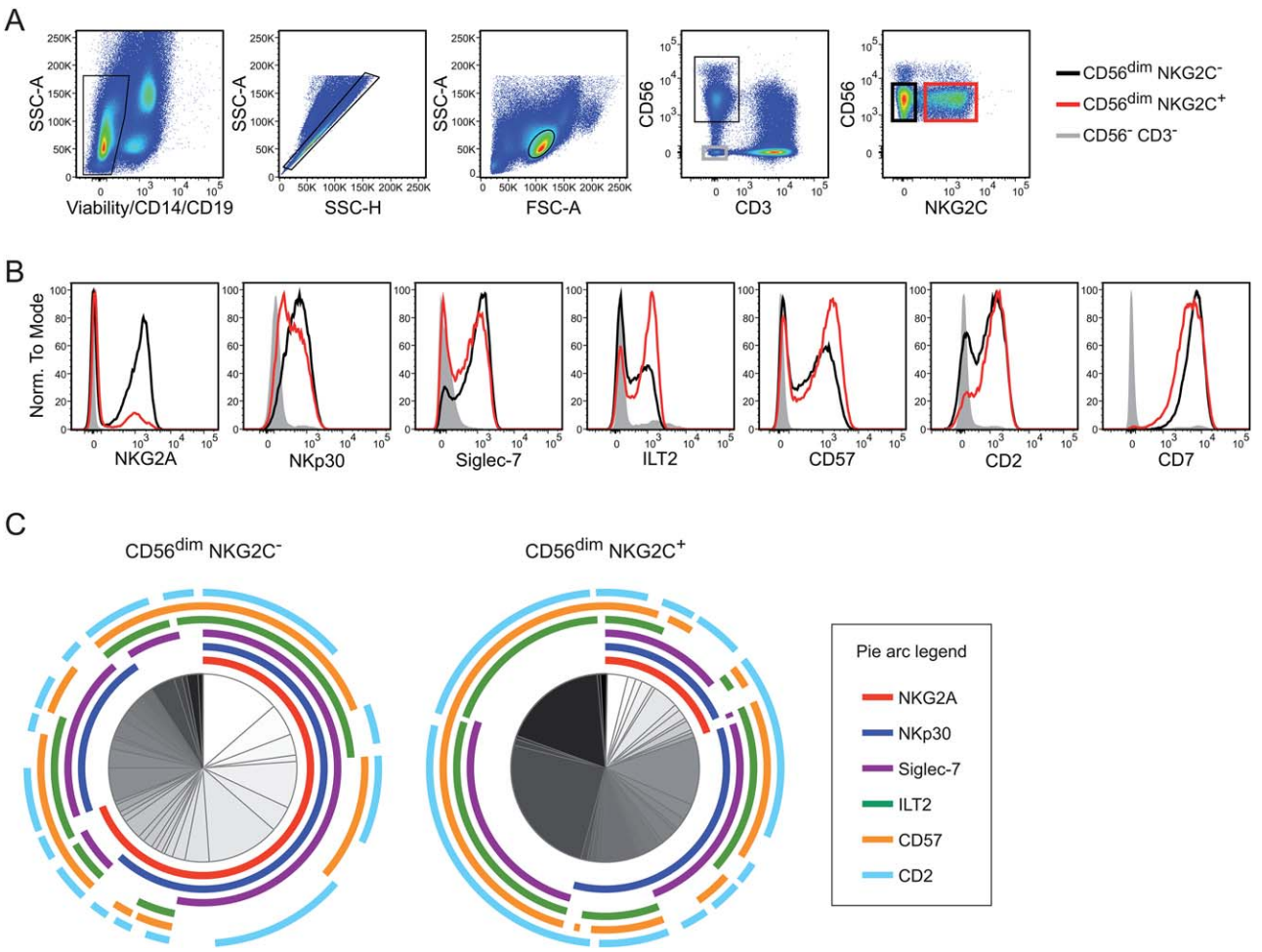

Figure 1. Gating strategy and exemplary performance of OMIP-039. Cryopreserved PBMC were thawed and stained with the complete panel as described in the Supporting Information. (**A**) Initial gating strategy to successively exclude dead cells, $CD14^+$ monocytes, $CD19^+$ B cells, and doublets while including large lymphocytes based on forward and side scatter. T and NKT-like cells are excluded by gating on $CD56^+$ $CD3^-$ cells and, finally, $CD56^{dim}$ NK cells are sub-divided based on the expression of NKG2C. (**B**) Differential expression of surface receptors on $CD56^{dim}$ $NKG2C^-$ (black lines) and $CD56^{dim}$ $NKG2C^+$ (red lines) NK cells. $CD56^-$ $CD3^-$ lymphocytes serve as internal staining control for NK-cell receptors (grey filled histograms). (**C**) Assessing the co-expression pattern of the surface markers allows detecting the presence of $NKG2A^-$ $NKp30^-$ $Siglec\text{-}7^-$ $ILT2^+$ $CD57^+$ $CD2^+$ cells within the $NKG2C^+$ population.

Table 2. Reagents used in OMIP-039

SPECIFICITY	FLUOROCHROME	CLONE	PURPOSE
Dead cells	Aqua	–	Viability (dump)
CD14	BV510	M5E2	Exclusion (dump)
CD19	BV510	HIB19	Exclusion (dump)
CD3	PE-Cy5	UCHT1	Exclusion
CD56	PE-Dazzle594	HCD56	NK cells
NKG2C (CD159c)	PE	REA205	Adaptive NK
NKG2A (CD159a)	PE-Vio770	REA110	Conventional NK
NKp30 (CD337)	eFluor450	AF29–4D12	Conventional NK
Siglec-7 (CD328)	APC-Vio770	REA214	Conventional NK
ILT2 (CD85j)	APC	HP-F1	Adaptive NK
CD57	Purified	TBO1	Adaptive NK
Mouse IgM	BV605	RMM-1	Detection of CD57
CD2	PerCP-Cy5.5	RPA-2.10	Adaptive NK
CD7	BV786	M-T701	Conventional NK

Human adaptive NK cells are confined to the $CD3^-$ $CD56^{dim}$ population and have so far not been reported for the $CD56^{bright}$ subset (6,8). Additionally, the majority of adaptive NK-cell populations express NKG2C as a primary activating receptor (6). Thus, after gating on $CD56^{dim}$ $NKG2C^-$ and $CD56^{dim}$ $NKG2C^+$ NK-cell populations, the expression of characteristic surface markers can be used to assess phenotypic differences (Figures 1A and 1B). Along the process of NK-cell differentiation (16–18), adaptive $NKG2C^+$ NK cells were demonstrated to be enriched for $NKG2A^-$ and $CD57^+$ cells and are consequently considered terminally differentiated (8,19). The initial description of adaptive $NKG2C^+$ NK cells in HCMV-seropositive healthy individuals included reduced proportions of cells expressing the natural cytotoxicity receptor NKp30 (CD337) as well as increased frequencies of ILT2 (CD85j)-expressing cells (8). In-depth phenotypical analyses have demonstrated that adaptive NK cells preferentially lack the inhibitory receptor Siglec-7 and that CD7 is expressed at lower levels per cell (6,11). Finally, analysis of large cohorts has revealed a slightly increased proportion of $CD2^+$ cells within adaptive NK-cell subsets (6,7,11). Importantly, CD2 not only serves as a marker, but is relevant for the (co-)activation of adaptive NK cells (7) and is therefore included in the panel.

Apart from comparing the expression of single receptors (Figure 1B), Boolean gates can be employed to assess the combinatorial expression pattern of the included markers using SPICE (20). By this approach, the presence of $NKG2A^-$ $NKp30^-$ $Siglec\text{-}7^-$ $ILT2^+$ $CD57^+$ $CD2^+$ cells within the $NKG2C^+$ population can be determined, thus detecting $NKG2C^+$ NK cells with a surface receptor phenotype prototypic for adaptive NK cells (Figure 1C). Details on this proposed detection strategy and a representative dataset on the performance of the panel are available in the Supporting Information.

After detection of adaptive NK cells, potential downstream applications include analysis of receptor usage, activation requirements, as well as expression of signaling molecules and transcription factors. Possible experimental settings and sets of respective reagents are illustrated in the Supporting Information.

Of note, the panel was established on samples from healthy individuals, in which the vast majority of NK cells in the peripheral blood express CD56, while $CD56^-$ $CD16^+$ cells are rare. However, as $CD56^-$ $CD16^+$ NK cells were reported to be increased in clinical settings such as chronic HIV or HCV infection (21–23), inclusion of CD16 in the gating hierarchy for the identification of $CD56^-$ NK cells should be considered. Further details as well as a potential reagent are provided in the Supporting Information.

Prior to panel design, reagents were titrated and compared with other commercially available conjugates and/or clones to define a core panel, which was successively combined with increasing numbers of antibodies in add-in experiments. FMO controls were used throughout panel optimization and can be used to guide setting of gate boundaries. Detailed information on the development and optimization of the panel are part of the Supporting Information. All reagents included in the final panel are listed in Table 2.

Similarity to Published OMIPs

OMIP-007 (24) and OMIP-029 (25) focus on the phenotypic analysis of human NK cells and OMIP-027 (26) is optimized to assess functional responses of NK cells. However, all three panels do not include marker combinations to discern between adaptive and conventional NK cells.

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Conflict of Interest

The authors declare no competing financial interests.

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