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OMIP-070: NKp46-Based 27-Color Phenotyping to Define Natural Killer Cells Isolated From Human Tumor Tissues

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- **Purpose and appropriate samples**

This 27-color panel has been validated and optimized to comprehensively profile natural killer (NK) cells isolated from human tumors using a collagenase Type II-based digestion protocol. We confirmed that detection of protein expression by antibodies used in our final panel was not affected during tissue digestion. During this evaluation process, we found that detection of CD56, a biomarker typically used to identify NK cells, was affected substantially by collagenase-based digestion. Thus, our panel is centered around expression of NKp46, which is sufficient to identify NK cells and not affected by the tissue collagenase digestion step. Our panel further includes biomarkers used to extrapolate NK-cell maturation, differentiation, migration, homing potential, and functional state. Our panel is intended to provide in-depth characterization of human NK cells isolated from tissues, which we specifically tested using oral squamous cell carcinomas tissues, but it is compatible with other tissues that can be dissociated with a collagenase Type II-based protocol.

- **Key terms**

high-dimensional cytometry; natural killer cells; human tumor; immunophenotyping; human tissue; human PBMCs

Background

Natural killer (NK) cells were first identified in the 1970s for their spontaneous cytotoxicity against target cells (1,2). Based on this ability to eliminate tumor cells without the need of prior immunization, it has been proposed that NK cells possess an ongoing preventive role to eliminate malignantly transformed cells and contribute to the control of hematologic malignancies or tumor metastasis (3,4). In addition to exerting direct cytotoxicity, NK cells are also able to produce cytokines and chemokines that modulate the local microenvironment and recruit additional immune cells (5-8). Together, these data suggest that NK cells could make significant contributions to antitumor immune responses in solid tumors (9). However, a more in-depth analysis of intra-tumoral NK cells is needed to further decipher how NK cells affect tumor growth. Of note, multiple studies have shown that the tumor progression and outcomes in humans correlate with the presence of NK cells at the tumor site (10-14) highlighting that these cells are of interest for the development of immunotherapeutic approaches in solid tumors (15-18).

One roadblock to study human NK cells isolated from tumors is the limited amount of tissue that is typically available from biopsies or tumor resections. It is thus of utmost importance to extract as much information as possible from the tumor sample on a single-cell level while minimizing further sample loss (Table 1).

Table 1. Summary table for application of this OMIP

PURPOSE	IMMUNOPHENOTYPING OF HUMAN NK CELLS IN TUMOR TISSUES
Species	Human
Cell types	Tumor tissues and peripheral blood. Oral squamous cell carcinoma tissues with donor-matched blood were used to profile NK cells with this 27-color panel
Cross-references	OMIP-007, OMIP-027, OMIP-029, OMIP-039, OMIP-056, OMIP-058, OMIP-064

The first critical step to profile infiltrating NK cells is to obtain a single-cell suspension yielding a high viability with minimal cell debris and preserved ability to detect critical cell surface antigens. Here, we used a well-established digestion method combining collagenase Type II and DNase. This collagenase Type II-based protocol has been used for nearly 20 years and yields an excellent percentage of viable mucosal mononuclear cells when processing human mucosal tissues (19-21).

The second critical step is to extract as much information as possible from this cell suspension. To that end, high parameter flow cytometry is particularly well suited. Indeed,

Table 2. Reagents used for OMIP-070

SPECIFICITY	ALTERNATIVE NAME	CLONE	FLUOROCHOME	PURPOSE
Lineage				
Dead cells	N.A.	N.A.	Blue	Viability
CD45	Protein tyrosine phosphatase receptor type C	HI30	BUV805	Lymphocytes
CD14	N.A.	M5E2	BV570	Monocytes
CD19	N.A.	SJ25-C1	BUV395	B cells
CD3	N.A.	UCHT1	BUV661	T cells
CD127	IL-7Rα	HIL-7R-M21	BV786	ILC
HLA-DR	MHC class II molecule	G46.6	APC-H7	Exclusion / activation marker
NK cell identification				
NKp46	CD335, NCR1	9E2	Biotin	NK cell marker and activating receptor
Streptavidin	N.A.	N.A.	BB630	N.A.
CD16	FcγRIII	3G8	BUV496	NK cell marker and ADCC
NK cell markers				
NKG2D	CD314, KLRK1	1D11	PE-Cy7	Activating receptor
CD244	2B4	2–69	BV421	Activating receptor
CD2	Cluster of differentiation 2	RPA-2.10	BV605	Co-stimulatory receptor
NKG2A	CD159a	REA110	PE	Inhibitory receptor
CD161	NKR-P1	DX12	BB700	Inhibitory receptor
TIGIT	T-cell immunoreceptor with Ig and ITIM domains	741182	BV711	Co-inhibitory receptor
CD69	Cluster of differentiation 69	FN50	BV650	Residency and activation
CD39	Cluster of differentiation 39	TU66	PE-CF594	Residency and activation
CD103	Integrin alpha E	Ber-ACT8	BV750	Residency
CX3CR1	Fractalkine receptor	2A9-1	BUV737	Migration
CD57	HNK-1, Leu-7	NK-1	FITC	Maturation and differentiation
CD27	Tumor necrosis factor receptor superfamily 7	M-T271	BB660	Maturation
CD11b	Integrin alpha M	ICRF44	PE-Cy5	Maturation and adhesion
CD38	Cyclic ADP ribose hydrolase	HIT2	BB790	Activation
Ki67	N.A.	Sola15	eFluor660	Proliferation
Granzyme B	N.A.	QA16A2	AF700	Cytolytic abilities
CD25	IL-2Rα	2A3	BUV563	Activation
CD56	Neural cell adhesion molecule (NCAM)	CMSSB	PECy5.5	Activation marker and NK-cell marker for peripheral blood

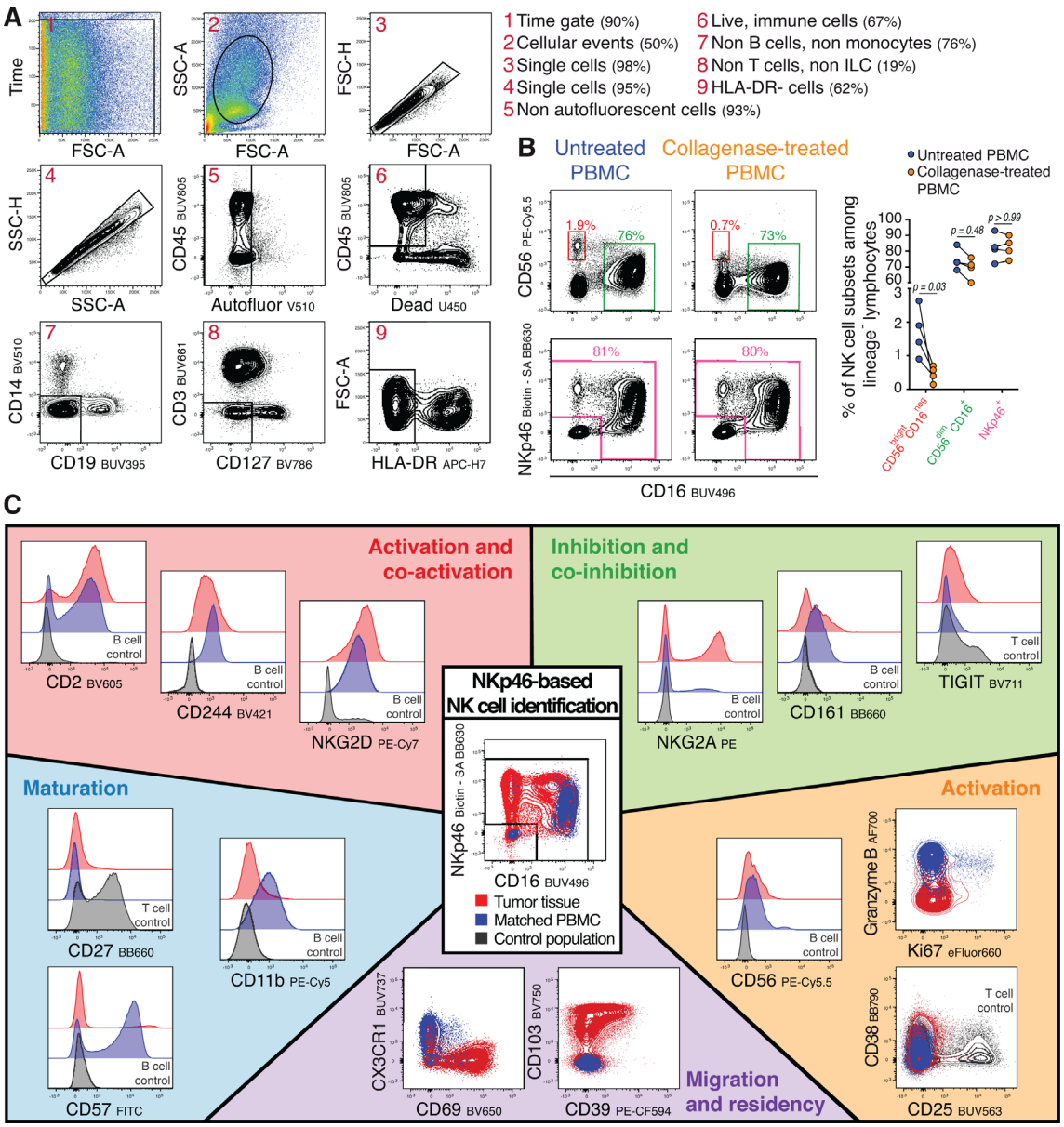

Figure 1. Gating strategy, NKp46-based approach and panel performance. (**A**) Gating strategy to identify NK cells in a single-cell suspension from a representative tumor tissue. Stable acquisition over time is monitored (1), followed by exclusion of debris (2), doublets (3, 4), and autofluorescent cells (5). NK cells were identified as live lymphocytes (6), non-CD14$^+$ monocytes (7), non-CD19$^+$ B cells (7), non-CD3$^+$ T cells (8), non-CD127$^+$ innate lymphoid cells 1 (ILC1) (8), and non-HLA-DR$^+$ cells (9). (**B**) To determine whether protein detection is affected by the collagenase Type II-based tissue digestion protocol, peripheral blood mononuclear cells (PBMC) from healthy donors were treated with 350 U/ml of collagenase Type II for 30 min at 37°C. The bivariate dot plots are representing the expression of CD56 and CD16 or NKp46 and CD16 from one healthy donor before or after collagenase digestion. The right graph shows the frequencies of each different highlighted population within the HLA-DR$^-$ cells as depicted in A ($n = 4$, Mann Whitney Test). (**C**) Overview of the 18 phenotypic molecules analyzed within the NKp46$^+$ cells of the tumor tissue (red) of a representative donor and its matched-peripheral blood (blue). Central bivariate dot plot shows the gate used to identify NKp46-expressing cells for both samples. Surrounding histograms or dot plots are showing the expression of the markers for both samples classified in the different categories: activation and co-activation markers, inhibition and co-inhibition markers, maturation markers, activation markers and migration and residency markers. As controls, both fluorescence minus one staining (FMOs) (Supporting Information Online Fig. S7), as well as reference populations, which were either CD19$^+$ B cells (serving as negative expression control) or CD3$^+$ T cells (serving as positive expression control) from the same tumor tissue, were used.

while cell loss can occur during staining and washing procedures, it is minimal during acquisition, with a current cell transmission efficiency greater than 95% for a standard fluorescence-based flow cytometer (22).

Here, we report the development and validation of a 27-parameter flow cytometry panel suitable to define the phenotype and activation status of NK cells isolated from human tumor tissues. We use a type II collagenase-based isolation

protocol, which ensures that the panel described here is useful and applicable for a range of human mucosal tissues and possibly others (20,23,24). All reagents included in the final panel are listed in Table 2.

In order to identify NK cells in tumor tissue, we applied the gating strategy depicted in Figure 1A. This gating strategy was designed to ensure that other immune populations such as B cells, T cells, and monocytes, could also be enumerated. NK cells were gated as live hematopoietic cells (Dead$^-$, CD45$^+$), non B cells (CD19$^-$), non-monocytes (CD14$^-$), non T cells (CD3$^-$), non-innate lymphoid cells (ILC) (CD127$^-$), and HLA-DR$^-$ cells (Fig. 1A). Of note, HLA-DR is a common surrogate for immune activation on lymphocytes (25) that has been reported to be expressed by a fraction of NK cells (26-28). However, CD16 expression by HLA-DR$^+$ cells could also point to the identification of non-classical CD14$^-$ monocytes (29). We observed that a small fraction of NKp46$^+$ cells expressed HLA-DR in the blood and the tumor tissues but kept HLA-DR as a lineage marker. Future single-cell RNA sequencing studies will help determine the nature of these cells and dictate if HLA-DR should be included in the gating strategy or as an activation marker.

Human peripheral blood NK cells are typically defined by the relative expression of two different surface markers: CD56 (NCAM), an adhesion molecule that has also been shown to be involved in NK cell activation (30), and CD16, the low-affinity Fc receptor known as FcyRIII that binds to the Fc domain of IgG antibodies and induces antibody-dependent cellular cytotoxicity (ADCC) upon binding (31). We found that the detected level of CD56 was decreased during the isolation process required to fully dissociate tumor tissue and obtain a single cell suspension (Fig. 1B). This sensitivity to a commonly used collagenase Type II-based digestion protocol (20) highlighted that CD56 was not an ideal marker to clearly define NK-cell subsets in tissues. Likewise, it might also explain the increased frequency of CD56^{dim} CD16$^-$ NK cells that has been observed in human tissues (32-34). As an alternative we have decided to use NKp46, a member of the highly conserved natural cytotoxicity receptor (NCR) family of NK-activating receptors, which has been proposed to be a unique marker to define NK cells across species (35) in context of other lineage markers.

We then compiled a set of 18 biomarkers, which are highly relevant for assessing NK-cell phenotype and function. First, as NK-cell activation is regulated by the integration of signals from a wide array of receptors (36), we included the activating receptors NKG2D and CD244, and the inhibitory receptors CD161 and NKG2A. We also included CD2, a cell adhesion molecule that has been shown to be relevant for activation of NK cells and memory formation (37,38).

We then tested several proteins with inhibitory properties such as PD-1, Tim3, and TIGIT (39), but only included TIGIT in the final panel (40). We found that Tim3 expression was sensitive to collagenase treatment, and, in line with a recent publication we did not observe PD-1 expression by NK cells (41).

We also included markers used to define NK-cell differentiation (CD57, CD11b, CD27) (42-44), activation (CD38, CD25) (45,46), and cytotoxic potential (Granzyme B). Ki67 was included as an indicator of cell proliferation. Finally, to assess migratory potential and tissue residence, CD69, CD103, and CX3CR1 were included in this panel along with CD39, a marker of chronic antigen stimulation (18).

The performance of the NK panel is displayed in Figure 1C. We found that this combination of markers enabled a thorough phenotypic characterization of NK cells within tumor tissues limited in both size and cell number. Of note, although our panel was developed primarily to assess NK cell phenotype, the expression of most markers could be assessed for CD3$^+$ T cells, providing additional valuable biological insight (Supporting Information Online Fig. S9).

Human Samples

Following approval from the ethical committee of the Institutional Review Board (IRB) of the Fred Hutchinson Cancer Research Center (Seattle USA), fragments of fresh tumor tissue as well as peripheral blood from the same donor were obtained from patients undergoing surgery for a head and neck squamous cell carcinoma in the Department of Otolaryngology, Head and Neck Surgery and Oncology, University of Washington, Seattle, USA.

Similarity to Other Published OMIPs

While there are other OMIPs that allow for a broad immunophenotyping of NK cells, to date, there is no OMIP specifically designed to deeply profile NK cells isolated from tumor tissues. We included the necessary concomitant assessment of how the tissue digestion protocol affects detection of surface proteins. OMIP-007 (47), OMIP-029 (48), OMIP-056 (49), OMIP-058 (50), and OMIP-064 (51) focus on phenotypic analysis of human NK cells from peripheral blood, OMIP-027 (52) is optimized to assess functional responses of NK cells, and OMIP-039 (53) allows distinguishing adaptive from conventional NK cells.

This 27-parameter OMIP is optimized for the identification and in-depth characterization of the NK cell infiltrate within collagenase-digested human tumor tissue and will be a helpful tool for the study of immune cell function in human tumors as well as other human mucosal tissues.

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Author Contributions

Marie Frutoso: Conceptualization; data curation; formal analysis; investigation; methodology; visualization; writing-original draft. **Florian Mair:** Conceptualization; formal analysis; investigation; methodology; visualization; writing-original draft; writing-review and editing.

Conflict of Interest

The authors declare no conflict of interest.

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