Flow cytometry

Panel design and analysis of natural killer cell populations using spectral unmixing on the Attune Xenith Flow Cytometer

Introduction

This application note presents a panel specifically designed to study natural killer (NK) cells. NK cells are a group of immune cells responsible for identifying and eliminating infected or cancerous cells through the recognition of specific markers or their absence (Figure 1). This study aims to investigate various markers related to NK cell activation, inhibition, and maturation. The spectral panel consists of 25 markers and is optimized for use with the Invitrogen™ Attune™ Xenith™ Flow Cytometer, which offers rapid analysis of a large number of cells. We provide an overview of the panel design, the markers included, and their significance in NK cell research.

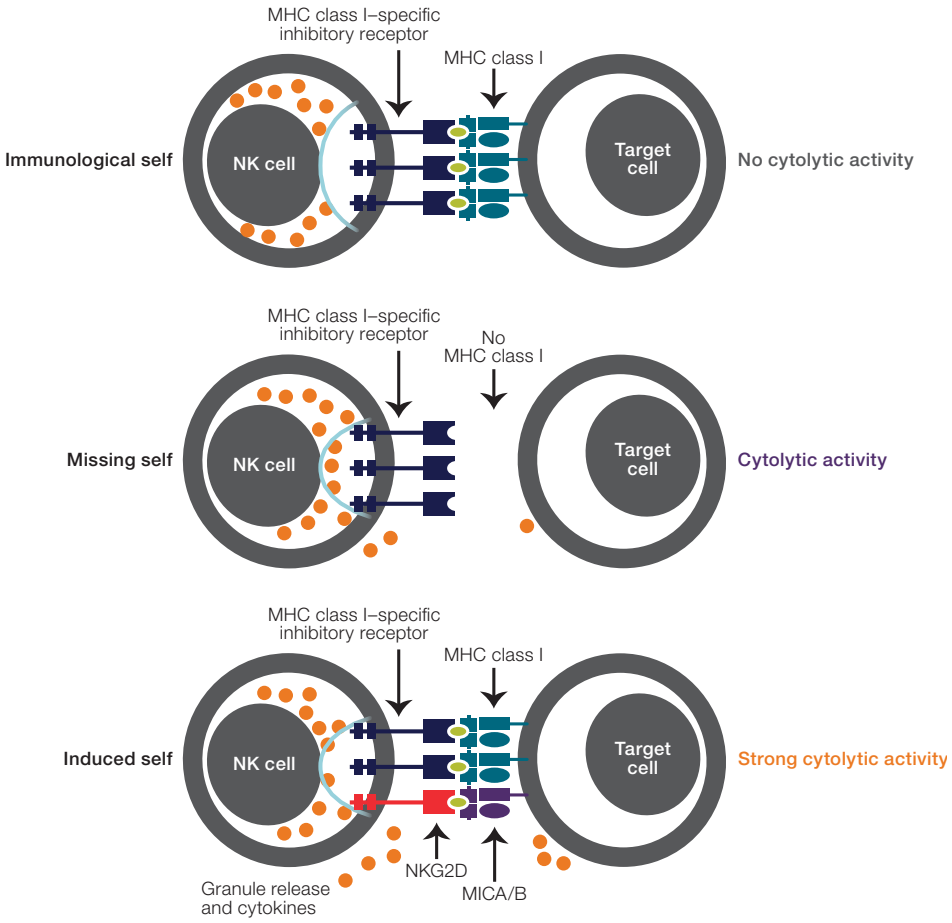

Figure 1. NK cells recognize the presence or absence of specific cell markers and react accordingly.

Flow cytometry analysis

The lymphocyte population is gated for singlets. Dead cells are excluded using Invitrogen™ LIVE/DEAD™ Fixable Near IR (876) viability dye, which utilizes infrared (IR) detection on the Attune Xenith Flow Cytometer. Further exclusion of unwanted cell types, such as T cells, B cells, HLA-DR–positive cells, and CD127-positive cells, allows for focused analysis of the NK population. Within the NK population, the maturation stages, including immature, mature, and terminal NK cells, are identified by CD56 and CD16 markers.

Panel design

With the tools provided in Invitrogen™ Sasquatch software, users can identify panel expansion opportunities. These tools include the spectral energy plot (Figure 2), similarity index, and complexity score values. Additionally, users may visualize the effects of unmixing on single-color controls to identify fluorophore pairs where overlap may influence data resolution.

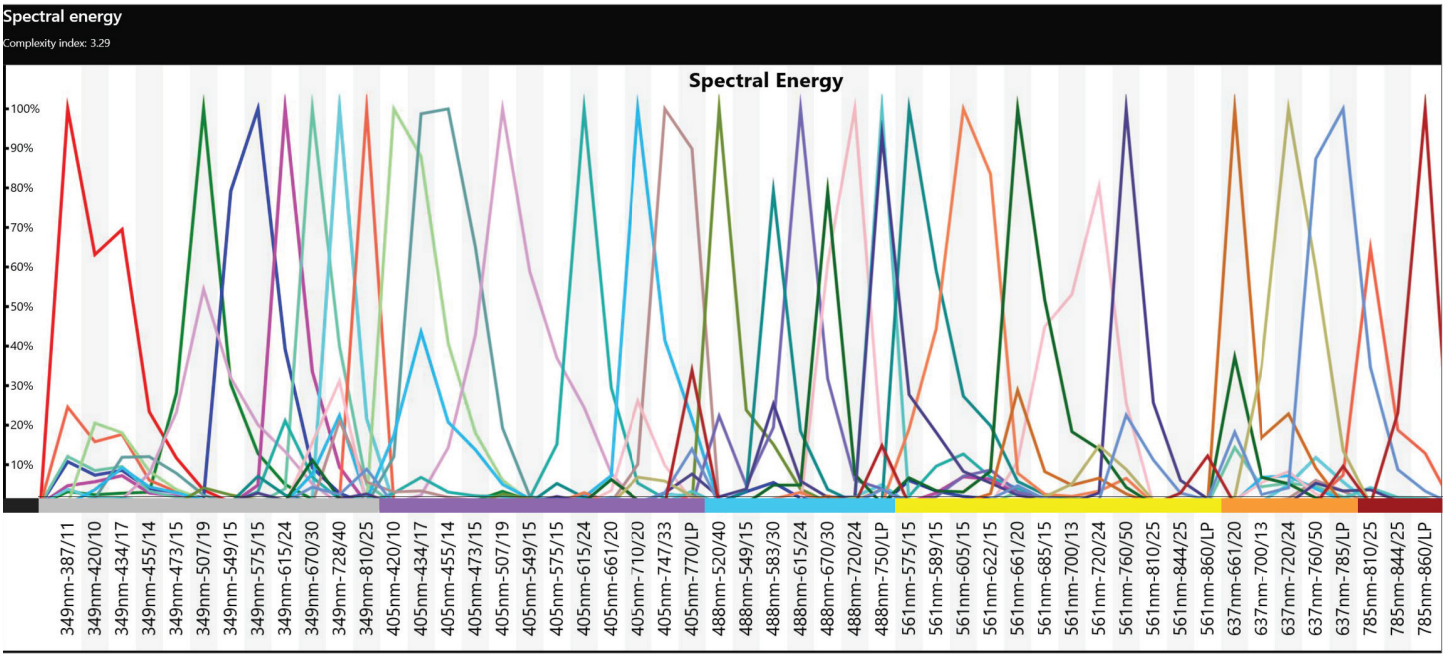

Figure 2. Sasquatch software tools for the Attune Xenith Flow Cytometer facilitate advanced panel design. In the spectral energy plot, the panel fluorophores are graphed against all detectors to identify areas with minimal overlap where the panel can be further expanded. A specific emission curve can be shaded by highlighting it in the Selected Fluorophores list in the Sasquatch software.

Table 1 presents the natural killer cell panel design grid, which shows selected fluorophores for each antigen and for differentiation of dead cells. For this assay, bead controls were used for all fluorophores and antigens with the exception of CD127 and HLA-DR. These fluorophores are mapped across detectors in the spectral energy plot in Figure 2.

Antigen expression analysis

The panel includes several pan-NK markers for which varying expression levels are expected across the NK populations evaluated. The expression of these markers is assessed using histograms representing the various maturation stages of NK cells. The panel includes additional pan markers such as CD11b, expressed on all circulating NK cells, and CD161, expressed to varying degrees in different NK cells.

Activation and inhibition marker analysis

Activation markers play a crucial role in elucidating the readiness and activity of NK cells. The panel includes markers such as NKG2D, NKG2C, CD2, and CD38, which exhibit different expression patterns across the maturation stages of NK cells. NKG2C, for example, is primarily expressed in mature NK cells, while NKG2D is expressed in almost all NK cell populations. In contrast, inhibitory markers, including NKG2A, KIR markers, and KLRG1, are analyzed using forward scatter of mature NK cells. Histograms demonstrate the expression of these markers in different maturation stages, highlighting their importance in NK cell populations.

To assess the functionality and responsiveness of the panel, cells were stimulated with a cytokine cocktail, as certain markers are not expressed on naive NK cells; these are stimulated overnight with a cytokine cocktail (IL-2, IL-15, and IL-21). CD69 and TIGIT are among these markers, which show significant shifts in expression upon stimulation, particularly in mature NK cells. NKP46, a pan-NK marker expressed to some degree in almost all NK cells, also exhibits notable shifts in expression following activation.

Table 1. Natural killer cell panel.

Laser and channel	Filter	Fluorophore	Antigen	Clone	Fluorescence-minus-one (FMO) controls
349 UV-1	387/11	Brilliant Ultra Violet™ 395	NKG2D	1D11	Yes
349 UV-6	507/19	Brilliant Ultra Violet™ 496	CD11b	ICRF44	Yes
349 UV-8	575/15	Brilliant Ultra Violet™ 563	CD56	NCAM16.2	
349 UV-9	615/24	Brilliant Ultra Violet™ 615	PD-1	EH12.1	Yes
349 UV-10	670/30	Brilliant Ultra Violet™ 661	KLRG1	Z7-205.rMab	Yes
349 UV-11	728/40	Brilliant Ultra Violet™ 737	NKp30	p30-15	Yes
349 UV-12	750/LP	Brilliant Ultra Violet™ 805	CD2	RPA-2.0	Yes
405 V-1	420/10	Brilliant Violet™ 421	NKG2C	134591	Yes
405 V-3	455/14	eFluor™ 450	CD57	TB01	Yes
405 V-5	507/19	Brilliant Violet™ 510	CD3	OKT3	
405 V-8	615/24	Super Bright™ 600	CD244	eBioC1.7	Yes
405 V-10	710/20	Super Bright™ 702	CD127	eBioRDR5	Yes
405 V-11	747/33	Brilliant Violet™ 750	CD69	FN50	Yes
488 B-1	520/40	FITC	KIRDL2/3	DX27	Yes
488 B-2	549/15	NovaFluor™ Blue 610-70S	HLA-DR	LN3	
488 B-6	720/24	PerCP-eFluor™ 710	TIGIT	MBSA43	Yes
488 B-7	750/LP	Real Blue™ 780	CD19	SJ25C1	
561 Y-1	575/15	PE	KIRDL1	124	Yes
561-Y-3	605/15	PE-Dazzle™ 594	NKG2A	S19004C	Yes
561 Y-5	661/20	PE-Cy®5	NKp46	9E2	Yes
561 Y-9	760/50	PE-Cy®7	CD161	HP-3G10	Yes
637 R-1	661/20	APC	CD226	11AB7.4	Yes
637 R-3	720/24	Alexa Fluor™ 700	CD38	HIT2	Yes
637 R-5	785/LP	APC-eFluor™ 780	CD16	eBioCB16	Yes
781 IR-3	860/LP	LIVE/DEAD™ Fixable Near IR (876)	Dead cells	N/A	Yes

Results

Utilizing the panel described above with the Attune Xenith Flow Cytometer's high acquisition rates (200 µL/min), the activation and maturation of NK cells was characterized. Immature, mature, and terminal NK populations were identified from human peripheral blood mononuclear cells (PBMCs), and surface marker expression of each of these subpopulations was compared (Figure 3). For instance, the expression profiles of NK cell subsets NKp30, NKp46, CD226, and CD244 were further investigated (Figure 4), as were the shifts in expression levels of pan-NK markers that occurred when cells were stimulated with human IL-2, IL-15, and IL-21 (Figure 5).

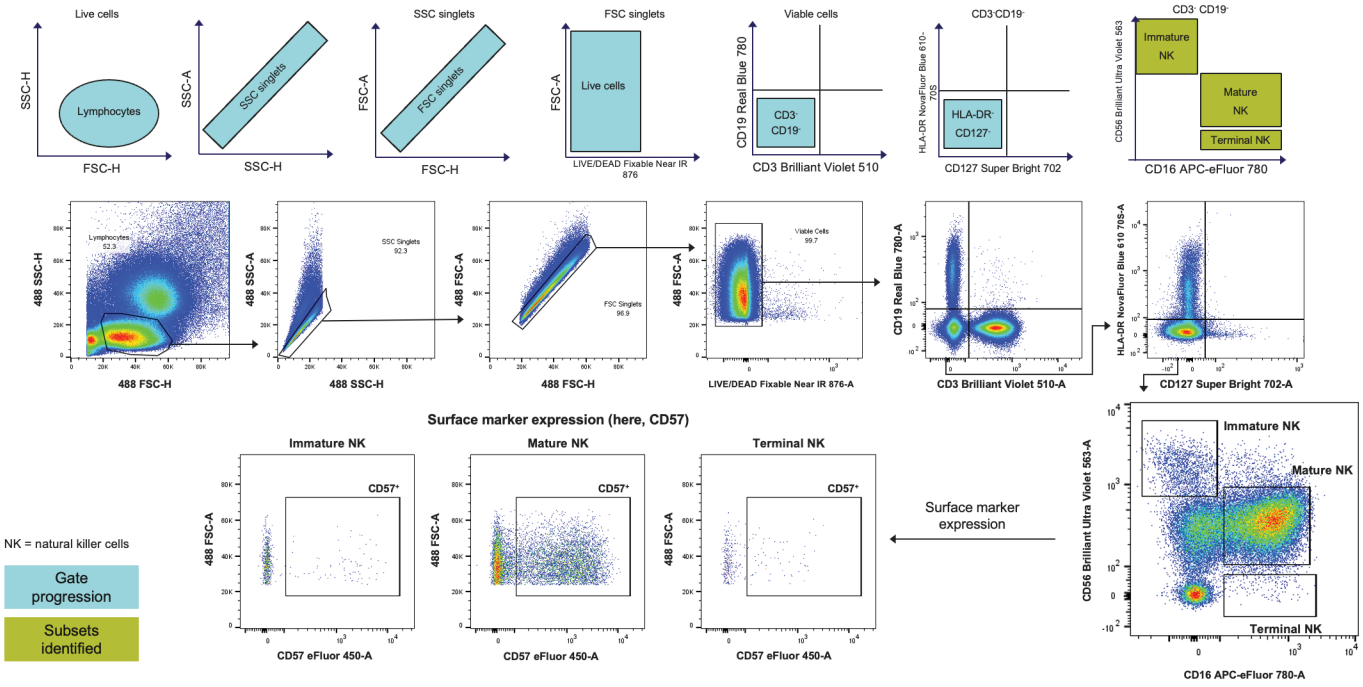

Figure 3. Gating scheme for 25-color natural killer cell panel. A 25-color flow cytometry panel was employed in the analysis of NK cells. Starting with the identification of viable lymphocytes, sequential gating steps were applied to exclude doublets, dead cells, and non-NK cell populations. The final gated population represents the NK cells, characterized by specific surface markers. Each color corresponds to a different fluorophore-conjugated antibody used in the panel, enabling the precise identification and analysis of NK cell subsets.

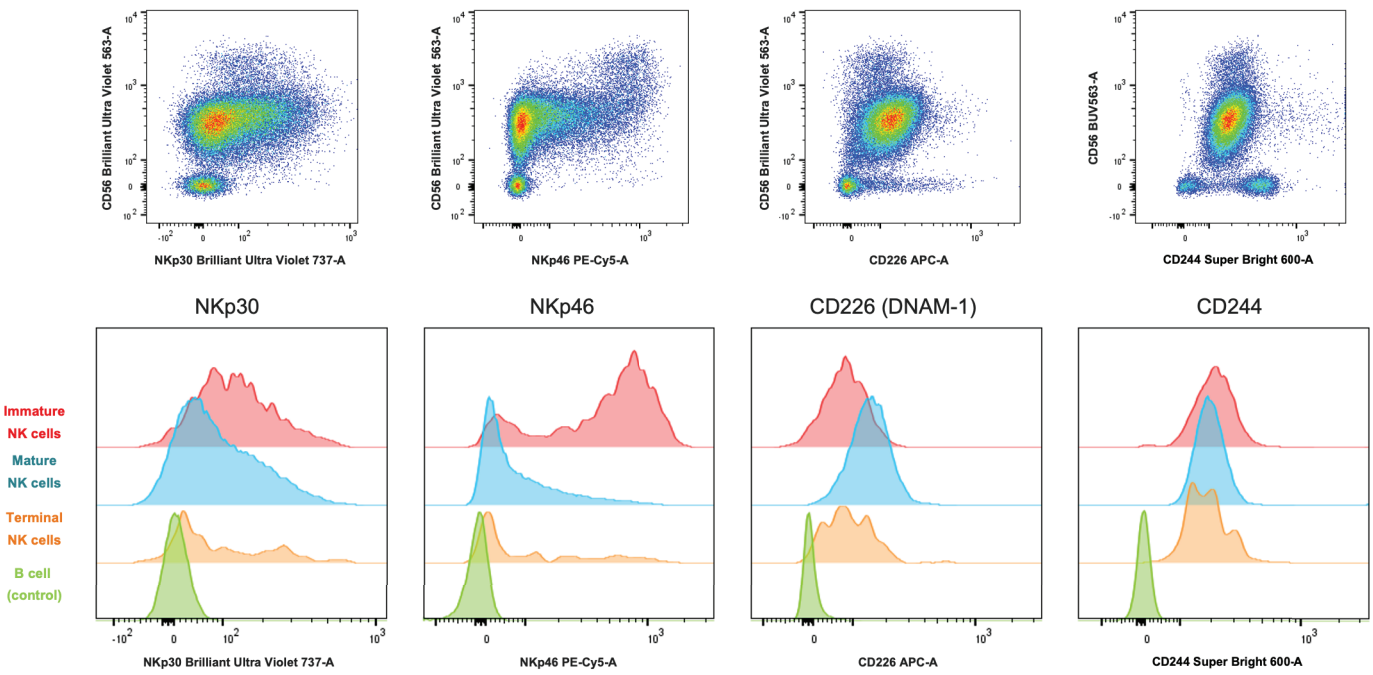

Figure 4. Pan-NK markers. Natural killer cell expression profiles of NKp30, NKp46, CD226, and CD244 can be further investigated between cell subsets.

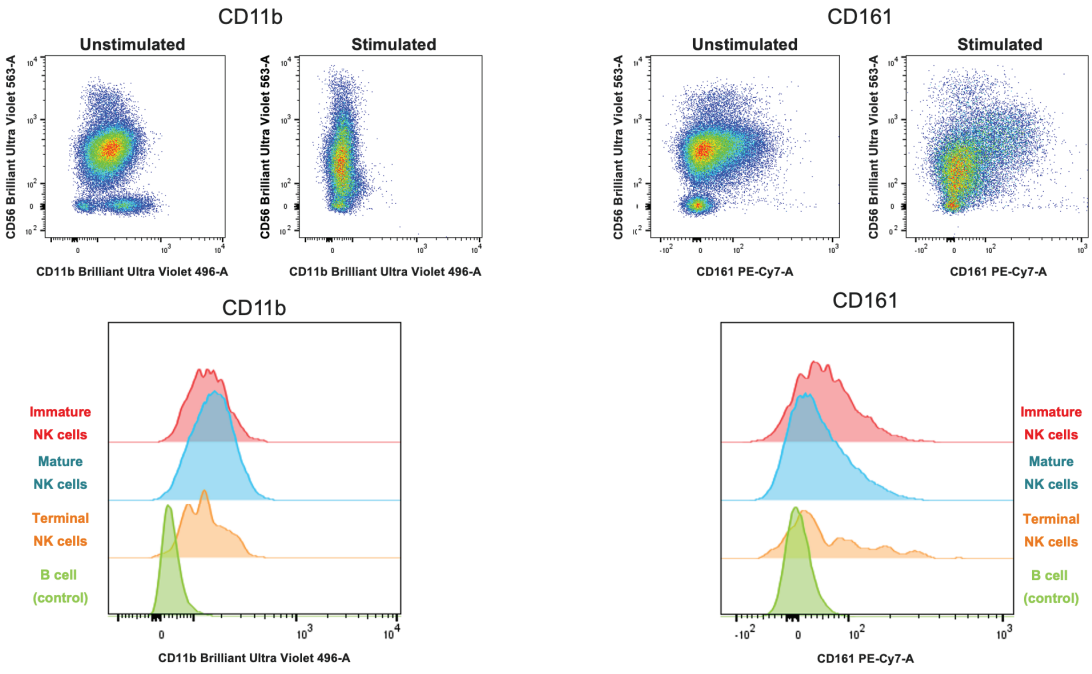

Figure 5. Pan-NK markers shift after cell stimulation. Expression level of CD11b or CD161 changes when NK cells are stimulated overnight with IL-2 at 100 IU/mL, IL-15 at 10 ng/mL, and IL-21 at 25 ng/mL.

Several markers of cellular activation were also investigated on immature, mature, and terminal populations. These markers are important for NK effector functions, and many increase in expression as the NK cells mature (Figures 6 and 7). In conjunction with activation markers, we also explored inhibitory antigen expression on the various NK cell populations, which will also change as cells mature and respond to cytokines or other biological stimuli.

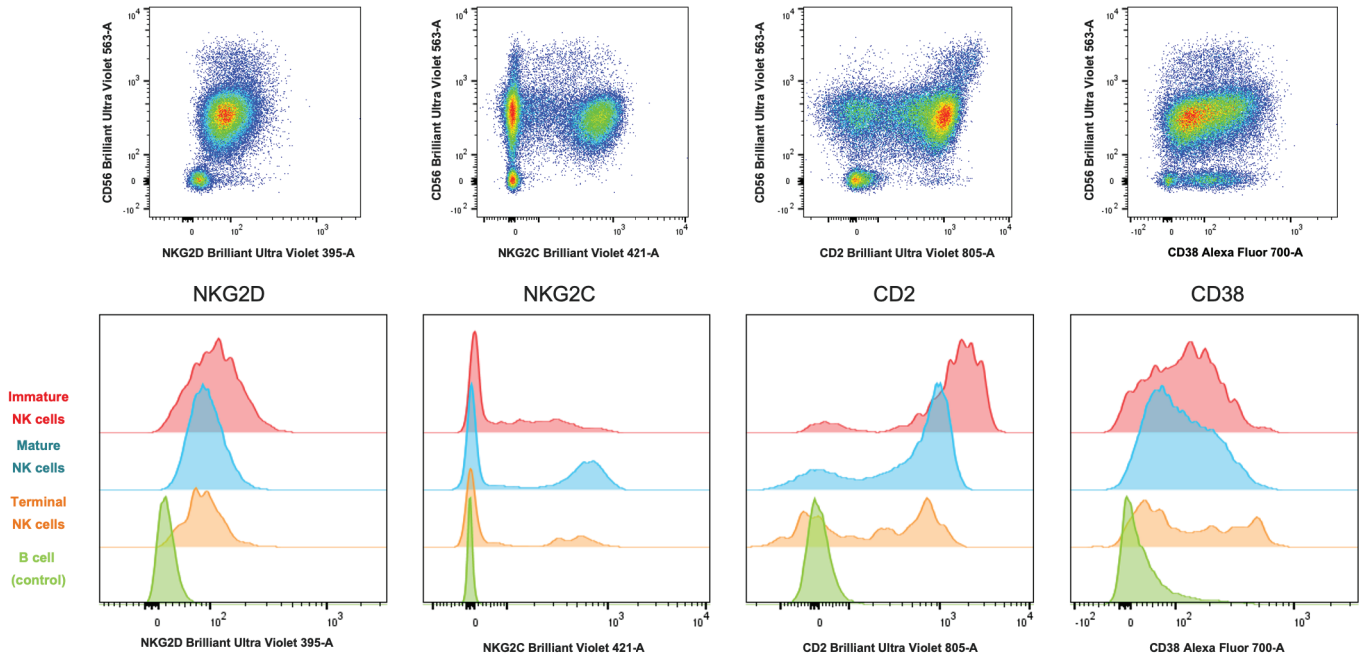

Figure 6. Activation markers in NK populations. Varied levels of maturation antigens are expressed in mature NK cell populations. The expression of markers such as NKG2C, CD2, and CD38 may be tracked during viral infections or activation by tumor cells.

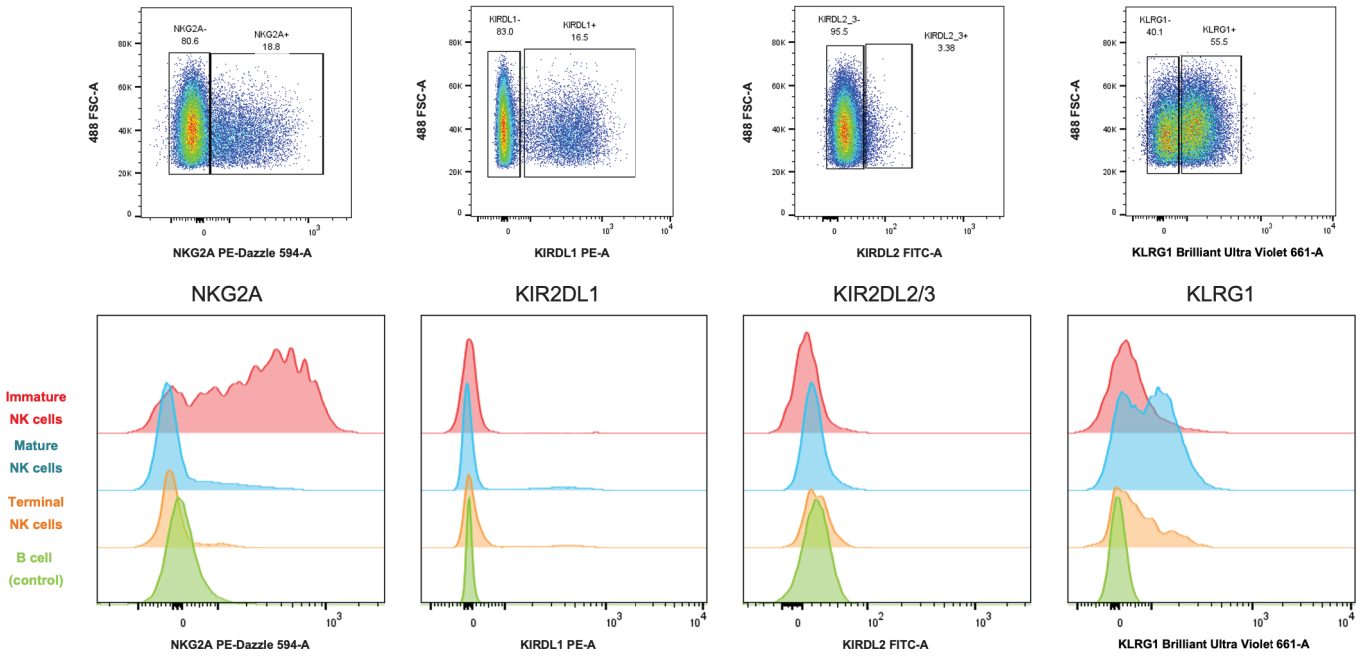

Figure 7. Inhibitory antigens in NK cell populations. Expression levels of NKG2A, KIR2DL1, KIR2DL2/3, and KLRG1, which regulate NK cell activation and function in response to tumors and viral infections, are shown.

Figure 8 displays the reaction of NK cell activation markers to stimulation of PBMCs. In NK cells, the level of expression can vary between populations at different levels of maturation, as demonstrated by 1D analysis of different markers upon stimulation.

The sensitivity of the Attune Xenith Flow Cytometer and the NK panel also allowed for the detection of rare events without the need for concentrating samples (Figure 9). High sample throughput reduced time-to-results without compromising population resolution. The sensitivity of detection helped ensure robust data resolution, enabling the differentiation of even rare populations in high-complexity panels. In Figure 9, antigen expression was evaluated in human NK cells that were incubated with or without an IL-2, IL-15, and IL-21 cytokine cocktail for a duration of 48 hr. The Attune Xenith Flow Cytometer demonstrated strong sensitivity in detecting changes across different stimulation procedures, helping to ensure reliable and accurate characterization of these rare-cell subsets. These results highlight the potential of the panel for conducting in-depth analysis and gaining a deeper understanding of NK cell subsets.

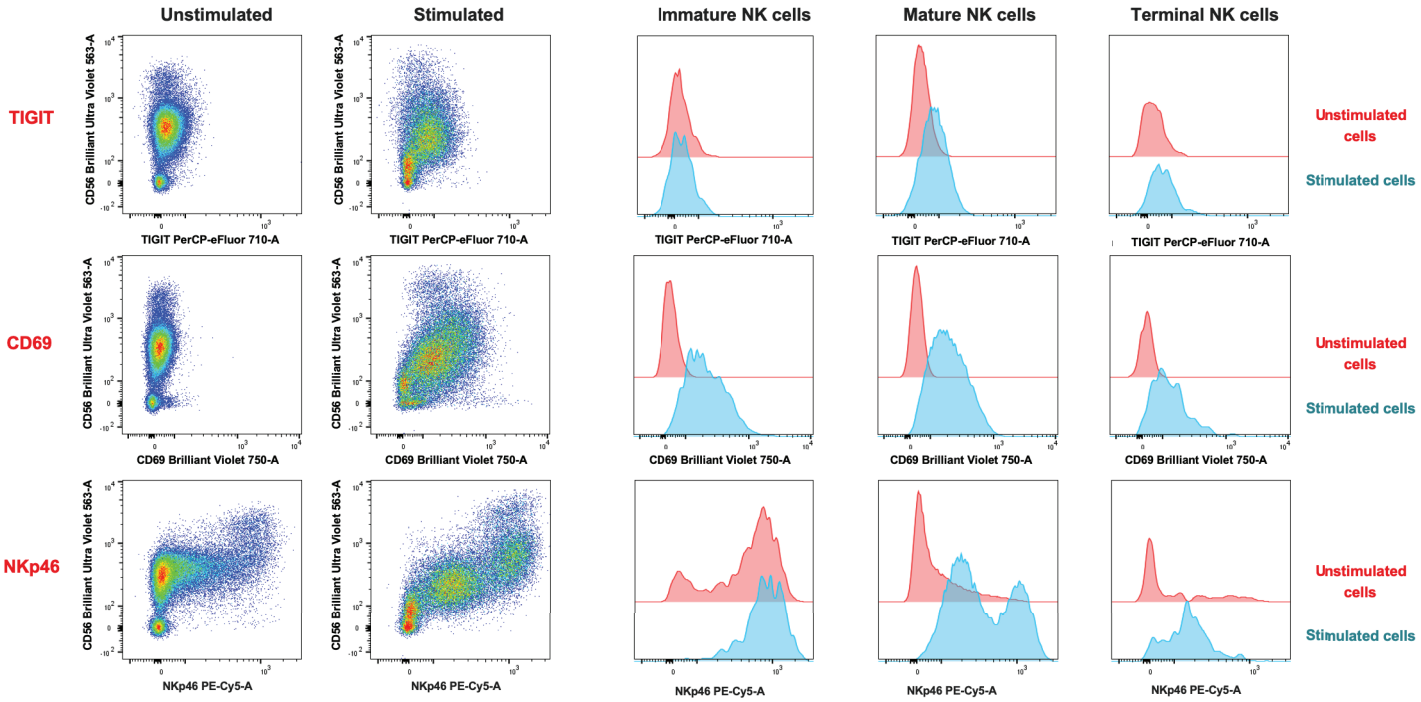

Figure 8. Expression of activation antigens in NK cells. When PBMCs are stimulated, NK cells display diverse expression of activation markers such as TIGIT, CD69, and NKp46. The levels of expression may vary between immature, mature, and terminal NK cells.

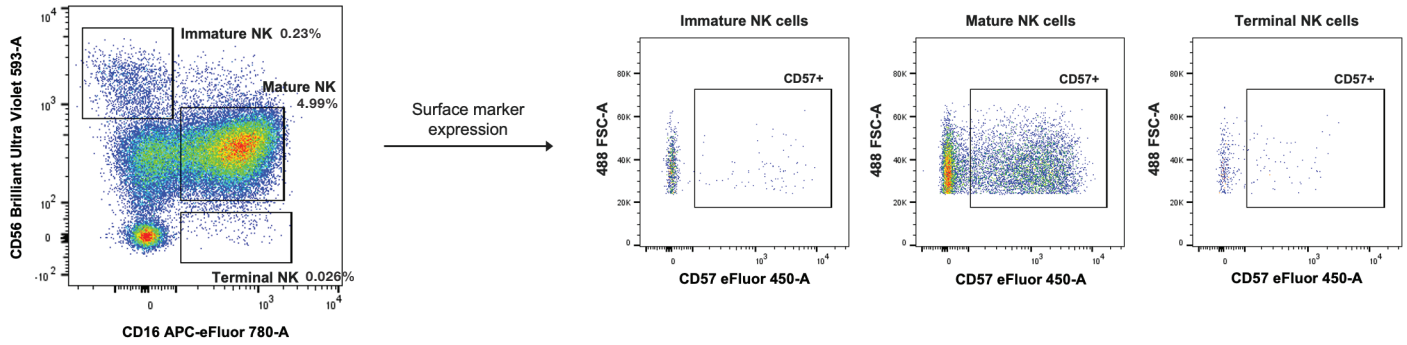

Figure 9. Precision in rare-event detection using a 25-color NK cell panel. Over a million events were rapidly collected (200 µL/min) to analyze NK cells from immature, mature, and terminal NK populations. Percentages of these populations as a portion of total cell count are shown in the first panel.

Discussion

Spectral flow cytometry has allowed for the expansion of panel size for a deeper investigation of populations of interest. Here we used 25 colors to perform an in-depth analysis of various NK populations from human PBMCs. Expression of key markers in the immature, mature, and terminal NK populations was analyzed to elucidate characteristics of NK activation and inhibition states. Various markers were used to label human PBMCs, both with and without cytokine stimulation, revealing significant changes in NK populations post-stimulation. Changes were observed in the expression of several important markers, including CD69, NKp46, and others, which align with general expectations of NK cell behavior under these conditions. This confirms the Attune Xenith Flow Cytometer's capability, along with the applied panel, to accurately investigate this important lineage in immuno-oncology research.

The Sasquatch software tools and unmixing results suggest that the panel has not yet fully maximized the detection capabilities of the system. As the panel evolves, further research should explore additional characterization of non-NKs within the PBMC samples and additional activation/inhibition markers within the NK subsets outlined here. This ongoing development will enhance the panel's utility for more comprehensive study of immune cell populations.

Conclusion

This application note has provided an overview of a comprehensive panel designed for the study of NK cells acquired on the Attune Xenith Flow Cytometer. The panel encompasses markers related to activation, inhibition, and maturation, allowing for a detailed analysis of NK cell populations.

This work highlights the potential of the panel and the Attune Xenith Flow Cytometer for conducting in-depth analysis and gaining a deeper understanding of NK cell subsets. Future plans include expanding the panel and addressing any queries related to its design, stimulation methods, marker selection, and fluorophore/antigen choices.

Methods

Supply information

Table 2 presents purchasing information for supplies necessary to perform the comprehensive NK cell panel analyses.

Table 2. Supplies for comprehensive NK cell panel analysis.

Product	Supplier	Cat. No.
Cell strainers (70 µm, nylon)	Corning	352350 or equivalent
Invitrogen™ Countess™ Cell Counting Chamber Slides and Holder, disposable	Thermo Fisher Scientific	T10282
Gibco™ Trypan Blue, 0.4% w/v	Thermo Fisher Scientific	15250061
Gibco™ Fetal Bovine Serum (FBS), Premium, heat-inactivated	Thermo Fisher Scientific	A5670502
Gibco™ AIM V™ Medium, liquid (research grade)	Thermo Fisher Scientific	12055091
Gibco™ CTS™ NK-Xpander™ Medium	Thermo Fisher Scientific	A5019001
Gibco™ Penicillin-Streptomycin (10,000 U/mL)	Thermo Fisher Scientific	15140148
Gibco™ PBS, pH 7.4	Thermo Fisher Scientific	10010023
Gibco™ Human IL-2 Recombinant Protein, PeproTech™	Thermo Fisher Scientific	20002
Gibco™ Human IL-15 Recombinant Protein, PeproTech™	Thermo Fisher Scientific	200-15-50UG
Gibco™ Human IL-21 Recombinant Protein, PeproTech™	Thermo Fisher Scientific	200-21-50UG
Invitrogen™ eBioscience™ Flow Cytometry Staining Buffer	Thermo Fisher Scientific	00-4222-26
Invitrogen™ eBioscience™ IC Fixation Buffer	Thermo Fisher Scientific	00-8222-49
Invitrogen™ UltraComp eBeads™ Plus Compensation Beads	Thermo Fisher Scientific	01-3333-42
Invitrogen™ Countess™ 3 Automated Cell Counter	Thermo Fisher Scientific	A49862
Human BD Fc Block™	BD Biosciences	564219
Invitrogen™ Brilliant Stain Buffer	Thermo Fisher Scientific	00-4409-42
Invitrogen™ CellBlox™ Plus Blocking Buffer	Thermo Fisher Scientific	C001T02F01
Human PBMCs, stored in vapor nitrogen phase	N/A	N/A

Table 3 displays detailed information on the necessary fluorophores.

Table 3. Fluorophore information.

Fluorophore	Antigen	Clone	Supplier	Cat. No.
Brilliant Ultra Violet 395	CD5	53-7.3	BD Biosciences	740206
Brilliant Ultra Violet 496	CD19	eBio1D3	Thermo Fisher Scientific	364-0193-82
Brilliant Ultra Violet 563	CD44	IM7	Thermo Fisher Scientific	365-0441-82
Brilliant Ultra Violet 615	CD11c	N418	Thermo Fisher Scientific	366-0114-82
Brilliant Ultra Violet 661	CD93	AA4.1	BD Biosciences	741574
Brilliant Ultra Violet 737	TCRgd	GL3	BD Biosciences	748991
Brilliant Ultra Violet 805	B220	RA3-6B2	Thermo Fisher Scientific	368-0452-82
Brilliant Violet 421	Siglec H	551	BD Biosciences	567815
Super Bright 436	F4/80	BM8	Thermo Fisher Scientific	62-4801-82
eFluor 450	CD21	eBio4E3	Thermo Fisher Scientific	48-0212-82
Brilliant Violet 480	CD62L	MEL-14	Thermo Fisher Scientific	414-0621-82
Brilliant Violet 510	CCR2	475301	BD Biosciences	747970
Brilliant Violet 570	Ly6G	1A8	BioLegend	127629
Super Bright 600	I-A/I-E	M5/114.15.2	Thermo Fisher Scientific	63-5321-82
Super Bright 645	CD25	PC61.5	Thermo Fisher Scientific	64-0251-82
Super Bright 702	IgD	11-26c	Thermo Fisher Scientific	67-5993-82
Brilliant Violet 750	CD4	GK1.5	BioLegend	100467
Super Bright 780	CD11b	M1/70	Thermo Fisher Scientific	78-0112-82
FITC	CD38	90	Thermo Fisher Scientific	11-0381-82
RB545	TCRb	H57-597	BD Biosciences	756204
NovaFluor Blue 610-70S	CD8	53-6.7	Thermo Fisher Scientific	M003T02B06-A
NovaFluor Blue 660-120S	CD205	205yekta	Thermo Fisher Scientific	M051T03B08
PerCP-eFluor 710	CD1d	1B1	Thermo Fisher Scientific	46-0011-82
Real Blue 780	CD24	M1/69	BD Biosciences	755892
PE	CD127	A7R34	Thermo Fisher Scientific	12-1271-82
RY586	CD27	LG.3A10	BD Biosciences	753482
PE-eFluor 610	IgM	II/41	Thermo Fisher Scientific	61-5790-82
PE-Cy5	CD3	17A2	Thermo Fisher Scientific	15-0032-82
PE-Cy5.5	CD49b	DX5	Thermo Fisher Scientific	35-5971-82
PE-Cy7	CD23	B3B4	Thermo Fisher Scientific	25-0232-82
PE/Fire 810	CX3CR1	SA011F11	BioLegend	149057
APC	CD335	29A1.4	Thermo Fisher Scientific	17-3351-82
NovaFluor Red 700	CD274	MIH5	Thermo Fisher Scientific	M036T03R03-A
Alexa Fluor 700	CD45	30-F11	Thermo Fisher Scientific	56-0451-82
APC-eFluor 780	Ly6C	HK1.4	Thermo Fisher Scientific	47-5932-82
APC-Fire 810	TER-119	TER-119	BioLegend	116264
LIVE/DEAD Fixable Near IR (876)	Dead cells	–	Thermo Fisher Scientific	L34982

Reagent preparation

Note: All reagents should be used at 4ºC unless otherwise noted or directed by the manufacturer.

1. LIVE/DEAD working solution:
    a. Prepare LIVE/DEAD Fixable Near IR 876 dye per manufacturer directions.
    b. Prepare working solution by adding 1 µL of LIVE/DEAD Fixable Near IR 876 suspension to 49 µL of 1X PBS, and pipette gently up and down to homogenize.
2. Prepare cytokine aliquots as directed by the manufacturer, storing at –80°C until use.
    a. Final concentrations added to the medium of choice will be 100 U/mL IL-2, 10 ng/mL IL-15, and 25 ng/mL IL-21.
3. Prewarm AIM-V Medium in a 37°C water bath.
4. If cell stimulation is to be utilized:
    a. Prepare complete NK-Xpander Medium with 5% heat-inactivated FBS and 100 U/mL penicillin-streptomycin.
    b. Prewarm in 37°C water bath.

PBMC thawing

1. Add tightly capped cryopreserved PBMC vials to a 37°C water bath for ~90 sec, then gently invert 3–4 times every 15–20 sec until vial is ~75% liquid.
2. Remove from water bath and wipe outside of vial to remove any remaining water.
3. For each vial of cryopreserved PBMCs (~1 mL volume, ~5 x 10^7 cells):
    a. Gently pour PBMCs into a 50 mL conical tube, and add 10 mL prewarmed AIM-V Medium.
    b. Slowly add 2 mL AIM-V Medium to each PBMC vial and pour into the same 50 mL conical tube used in step 3a. Repeat.
    c. Centrifuge at 400 x *g* for 5 min. Discard supernatant by gently pouring off.
    d. Resuspend PBMCs in 1X PBS by gently pipetting up and down (cells can be resuspended in any volume between 1 and 10 mL of PBS, depending on the desired concentration).
    e. Gently invert cell suspension several times to homogenize, and record concentration and viability with the Invitrogen™ Countess™ 3 Automated Cell Counter.

    Note: If viability is <90%, preparing another sample of cells from a fresh vial is recommended.

Cell stimulation

Cells are stimulated for 48 hr in an IL-2, IL-15, IL-21 cocktail. After 48 hr stimulation, cells are collected and stained as described in this and the next subsection. We recommend collecting an unstained sample of the stimulated cells, as they will have a slightly different autofluorescence (AF) profile than unstimulated cells and can be used as a separate negative control and AF parameter in unmixing experiments.

1. Divide cell suspension for cell stimulation.
    a. Aliquot appropriate volumes of cell suspension to separate conical tubes. A minimum of 9 x 10^6 live cells is recommended for stimulation treatment.
    b. Centrifuge at 400 x *g* for 5 min. Discard supernatant by gently pouring off.
    c. Resuspend the cells in each stimulation tube in NK-Xpander Medium to 3 x 10^6 live cells/mL.
    d. Resuspend cells in the negative control (no stimulation) tube in 1X PBS to 1 x 10^6 live cells/mL, and move directly to cell staining procedures.
2. To the cells to be stimulated, add IL-2 to 100 U/mL, IL-15 to 10 ng/mL, and IL-21 to 25 ng/mL final concentrations.
3. Aliquot to 6-well tissue culture plates with a minimum volume of 1 mL per well.
4. Incubate at 37°C for 48 hr.
    a. After 48 hr, transfer cell suspensions from culture plates to conical tubes, pipetting several times to homogenize.
    b. Gently invert cell suspension tube several times to homogenize, and record concentration and viability with the Countess 3 Automated Cell Counter.
    c. Centrifuge at 400 x *g* for 5 min. Discard supernatant by gently pouring off.
    d. Resuspend in 1X PBS to 1 x 10^6 live cells/mL.

Cell staining

1. PBMCs should be in 1X PBS at a concentration of 1 x 10^6 live cells/mL.
2. Gently invert the cell suspension tube several times to homogenize, and filter through a 70 µm cell filter into a fresh 50 mL conical tube.
3. Prepare heat-shocked cells for viability dye single-stain control:
   a. Add 1 mL (1 x 10^6 cells) to a microcentrifuge tube and place on heat block to incubate suspension for 5 min at 65°C. Remove from heat block and place on ice for 2–3 min.
   b. Add 500 µL of fresh PBMC suspension to cooled microcentrifuge tube from heat block incubation.
4. Separate sufficient PBMCs for unstained and single-color controls (no viability dye added).
5. To remaining suspension, add 1 µL L/D working solution per 1 mL of PBMCs, pipetting gently several times to homogenize.
6. Incubate all suspensions 30 min at 4°C, protected from light.
7. Centrifuge at 400 x *g* for 5 min at 4°C and discard supernatant, drawing off residual fluid with brief contact against an absorbent wipe.
8. Resuspend each cell pellet to 1 x 10^6 cells/mL in eBioscience Flow Cytometry Staining Buffer, pipetting gently to homogenize.
9. Block each cell suspension by adding 20 µL Human BD Fc Block™, 10 µL Invitrogen™ Brilliant Stain Buffer, and 5 µL Invitrogen™ CellBlox™ Plus Blocking Buffer per every 10^5 cells (i.e., 100 µL of resuspended pellet) in suspension. Gently vortex to homogenize.
10. Incubate for 15 min at 4°C, protected from light.
11. Transfer 100 µL of blocked cells to individual tubes.
    a. Samples will be the bulk PBMCs stained with LIVE/DEAD Fixable Near IR 876 dye.
    b. The viability single-color control will use the stained suspension containing heat-shocked cells.
    c. Unstained and all remaining single-color cell controls will use the unstained suspension.
12. Add an appropriate volume of each antibody to the relevant samples and controls. Gently vortex to homogenize.
13. Incubate for 30 min at 4°C, protected from light.
14. Wash samples by adding 1 mL of eBioscience Flow Cytometry Staining Buffer.
15. Centrifuge at 400 x *g* for 5 min at 4°C and discard supernatant, drawing off residual fluid with brief contact against an absorbent lab wipe.
16. Resuspend cell pellet in 1 mL eBioscience Flow Cytometry Staining Buffer, pipetting gently to homogenize.
17. Centrifuge at 400 x *g* for 5 min at 4°C and discard supernatant, drawing off residual fluid with brief contact against an absorbent lab wipe.
18. Resuspend cell pellet in 200 µL eBioscience Flow Cytometry Staining Buffer, pipetting gently to homogenize.
19. Proceed to immediate analysis or to cell fixation with eBioscience IC Fixation Buffer.

    Note: LIVE/DEAD Fixable viability dyes may be used on fresh samples and are stable with a wide range of fixatives. If an alternative viability dye is used, ensure that dead cell staining is consistent before and after fixation by testing this during assay development.

Bead control preparation

1. Prepare UltraComp eBeads Plus Compensation Beads per manufacturer directions.
2. Proceed to bead fixation with eBioscience IC Fixation Buffer, or to immediate analysis.

   Note: Bead controls should be treated in a manner similar to cell controls following staining. Thus, if fixation and/or permeabilization is performed on cell controls and samples, the same procedures should be completed on bead controls.

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