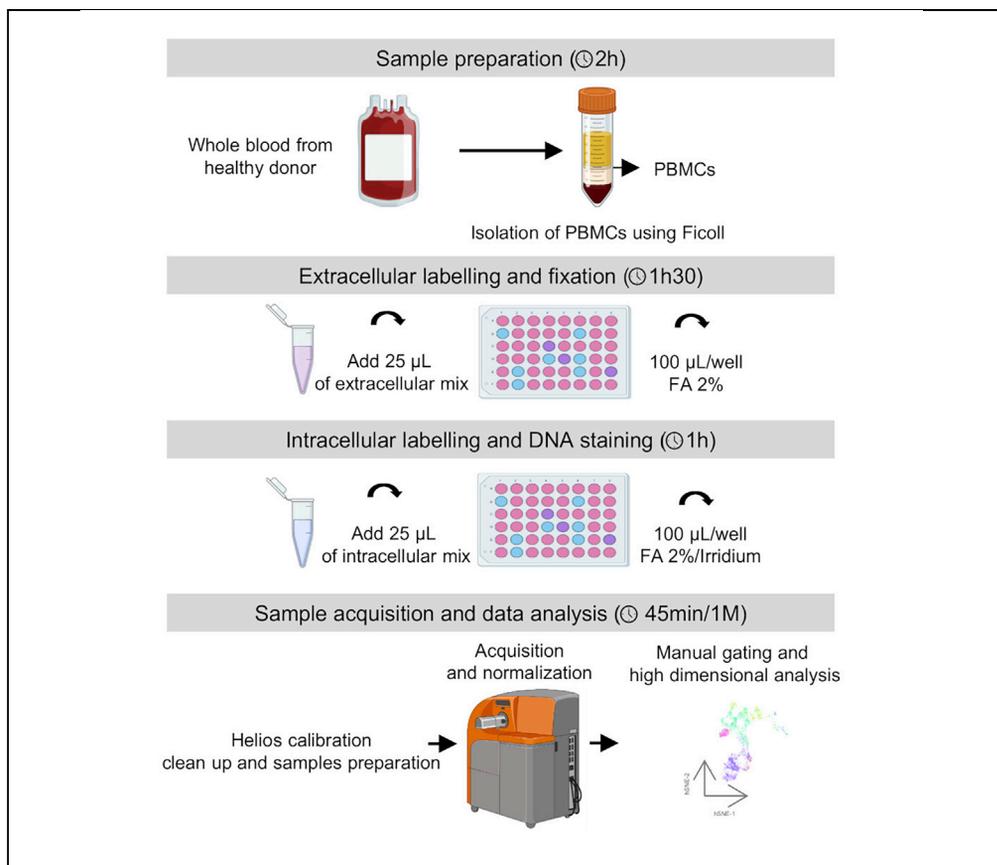


Protocol

High-throughput mass cytometry staining for deep phenotyping of human natural killer cells



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Highlights

Protocol for deep
phenotyping of
human NK cells by
mass cytometry

Sample staining with
metal-conjugated
antibodies

Sample acquisition
on a mass cytometer

High-dimensional
analysis using
machine learning
algorithms

This protocol details the step-by-step procedure for in-depth immune phenotyping of peripheral blood natural killer (NK) cells from clinical samples by mass cytometry. The protocol consists of three main steps: PBMC incubation with a mix of metal-conjugated antibodies for extracellular phenotyping followed by fixation, permeabilization and incubation with a mix of metal-conjugated antibodies for staining of intracellular proteins, and sample acquisition on a mass cytometer. High-dimensional analysis enables the visualization of NK cell subsets and their phenotypical characteristics.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol

High-throughput mass cytometry staining for deep phenotyping of human natural killer cells

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SUMMARY

This protocol details the step-by-step procedure for in-depth immune phenotyping of peripheral blood natural killer (NK) cells from clinical samples by mass cytometry. The protocol consists of three main steps: PBMC incubation with a mix of metal-conjugated antibodies for extracellular phenotyping followed by fixation, permeabilization and incubation with a mix of metal-conjugated antibodies for staining of intracellular proteins, and sample acquisition on a mass cytometer. High-dimensional analysis enables the visualization of NK cell subsets and their phenotypical characteristics.

For complete details on the use and execution of this protocol, please refer to Chretien et al. (2021).

BEFORE YOU BEGIN

Institutional permissions

△ CRITICAL: before collecting and working on human samples, it is mandatory to obtain permissions from the relevant institutions. In the present work, human samples from healthy volunteers were obtained from the EFS (Etablissement Français du Sang); authorization number for the conservation and preparation of products for non-therapeutic purposes, issued by the Ministry of Higher Education, Research and Innovation, is AC-2019-3428.

1. Antibodies conjugation to metal isotopes

⌚ Timing: 4 h

To design a panel, we first use available commercial antibodies. However, when the relevant clones are not available or when the antibody-metal combinations are not appropriate, we perform a custom conjugation. This protocol is adapted from the Maxpar Antibody Labeling User Guide.



1. Preload the polymer with lanthanide.
 - a. Thaw and quick spin X8 polymer tube then add 95 μL of L-Buffer to dissolve the polymer.
 - b. Add 5 μL of 50 mM Ln metal solution and incubate at 37°C in an incubator (do not use a water bath) for 30 min.
2. Partially reduce the antibody.
 - a. Add 300 μL with R-Buffer to the 50 kDa column, then add 100 μg (up to 400 μL) of purified antibody and centrifuge at 12,000 $\times g$ for 10 min at 20°C–22°C.
 - b. After discarding the flow-through, add 100 μL of freshly prepared 4 mM TCEP diluted in R-Buffer on the 50 kDa column. After a quick mix by pipetting, incubate at 37°C in an incubator for 30 min.
3. Purify the lanthanide-loaded polymer.
 - a. Add the polymer-metal complex to the labeled 3 kDa column containing 200 μL of L-Buffer and centrifuge at 12,000 $\times g$ for 25 min at 20°C–22°C.
 - b. Discard the flow-through then add 300 μL of C-Buffer and centrifuge at 12,000 $\times g$ for 30 min at 20°C–22°C.
4. Purify the partially reduced antibody.
 - a. Add 300 μL of C-Buffer to the 50 kDa column, and centrifuge at 12,000 $\times g$ for 10 min at 20°C–22°C.
 - b. Discard the flow-through, then add 400 μL of C-Buffer and centrifuge at 12,000 $\times g$ for 10 min at 20°C–22°C.

Optional: A second centrifugation can be added if step 3.b is not completed.

5. Conjugate the antibody with lanthanide-loaded polymer.
 - a. Discard the 3 kDa and 50 kDa column flow-through.
 - b. Resuspend the lanthanide-polymer complex in 60 μL of C-Buffer by pipetting and transfer the suspension (approximately 80 μL with residual volume) to the partially reduced antibody in the 50 kDa filter. Mix well by pipetting. The final conjugation volume should be approximately 100 μL . Incubate for 60 min at 37°C.
6. Wash and recover the metal-conjugated antibody.
 - a. Add 300 μL of W-Buffer to the 50 kDa column and centrifuge at 12,000 $\times g$ for 10 min. Discard flow-through and repeat this step 3 times.
 - b. Elute by adding 50 μL of W-Buffer to the 50 kDa column, pipet several times and invert the filter into a new collection tube then centrifuge for 2 min at 1,000 $\times g$. Repeat this step once.
 - c. Quantify the conjugated antibody using the NanoDrop spectrophotometer with the following settings: protein A280 module, IgG sample type option, 1 μL aliquot. Use W-Buffer as the blank. Conjugated antibody must be stored at 4°C in W-Buffer supplemented with 0.05% sodium azide at a final concentration of 0.5 mg/mL.

⚠ CRITICAL: This procedure is suitable for lanthanide but not cadmium isotopes.

⏸ Pause point: Metal-conjugated antibodies can be stored for a one year. However, a monitoring of the antibodies is carried out every month with a full panel on the same PBMCs control to check the absence of signal degradation.

Figure 1A.

II. Titrations of extracellular and intracellular antibodies

⌚ Timing: 5 h for sample preparation and 1 h for the acquisition on the mass cytometer

Antibody titration is critical to determine the optimal concentration of conjugated antibody. It allows an optimal staining, strengthens population identification, and decreases unspecific binding leading to background signal. The choice of optimal titer is based on good separation between the positive and negative

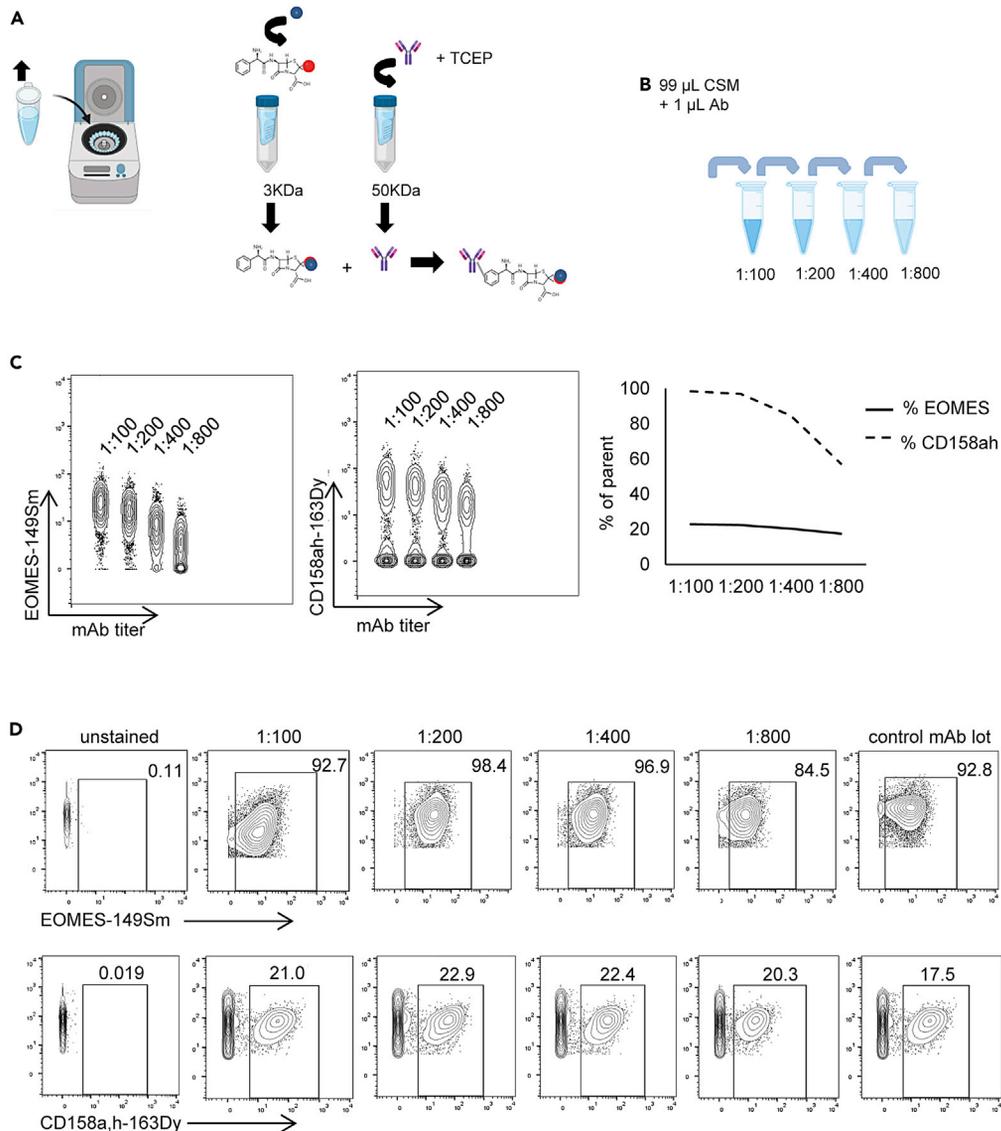


Figure 1. Antibodies titration

(A) Maxpar metal labeling procedure: antibody reduction, lanthanide loaded polymer and conjugation.

(B) Serial dilutions for antibodies titration.

(C and D) Overlay of serial dilution of contour plot, frequencies of positive cells, staining index.

population without spillover into the other channels. In-house metal-conjugated antibodies must be titrated for every new conjugation batch. A fraction of the current batch must be preserved to serve as a control. Ideally, the tests should be performed on samples of the same type as those to be analyzed.

For cell preparation please refer to [step-by-step method details](#) section. Rapidly cells were thawed, dead cells were discarded, counted and $3 \cdot 10^6$ cells were distributed in 96-well plate. Serial dilutions are prepared in cell staining medium (CSM) for extracellular antibodies and in perm wash for intracellular antibodies.

Note: In first intention, commercial antibodies are not titrated. Indeed, we start at the recommended concentration by the manufacturer (i.e 1 μ L antibody per 100 μ L cell suspension

containing 1–3.10⁶ cells). However, if a strong signal or a poorly focused separation between negative and positive populations is observed during the panel validation, commercial antibodies are also to be titrated.

Note: The proper titration of some epitopes requires prior stimulation in order to induce their membrane expression.

Optional: The titration of several antibodies together is possible provided that the following rules are respected: do not titrate antibodies in the same well if metal isotopes are located at N+1, N-1 and N+16 in order to avoid a possible cross-contamination in corresponding channels.

Figures 1B–1D.

Study design

7. Clinical Samples.

The mean age of healthy subjects (N = 22) recruited was 50.1 ± 11.3 with a sex ratio (M/F) of 1.2. We have not retained any particular criteria for healthy volunteer selection.

8. Reference Sample as Batch control.

A quality control is important for longitudinal studies where multiple batches will be run and compared. PBMCs isolated from a single healthy donor serve as reference sample. These PBMCs must be included in each batch of experiment. Thus, a correction can be applied to each batch with a batch effect correction algorithm.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD45-89Y (1:100)	Fluidigm	Cat# 3089003B
CD19-142Nd (1:400)	Fluidigm	Cat# 3142001B
CD45RA-143Nd (1:100)	Fluidigm	Cat# 3142001B
CD4-145Nd (1:400)	Fluidigm	Cat# 3145001B
CD8 α -146Nd (1:400)	Fluidigm	Cat# 3146001B
CD107a 151Eu (1:400)	Fluidigm	Cat# 3151002B
SIGLEC7-152Sm (1:100)	Fluidigm	Cat# 3152013C
CD27-155Gd (1:200)	Fluidigm	Cat# 3155001B
Tbet 161Dy (1:100)	Fluidigm	Cat# 3161014B
NKp46-162Dy (1:100)	Fluidigm	Cat# 3162021B
NKG2D-166Er (1:100)	Fluidigm	Cat# 3166016B
DNAM-1-171Yb (1:100)	Fluidigm	Cat# 3171013B
CD57-172Yb (1:1600)	Fluidigm	Cat# 3172009B
Granzyme 173Yb (1:200)	Fluidigm	Cat# 3173006B
CD56-176Yb (1:400)	Fluidigm	Cat# 3176001B
CD16-209Bi (1:100)	Fluidigm	Cat# 3209002B
CD3-115In (1:800)	Immunomonitoring lab*	#UCHT1
TCRV δ 2-141Pr (1:400)	Beckman Coulter	C65929
NKG2C-147Sm (1:100)	Miltenyi	130-122-278

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
TCRpanyδ-153Eu (1:400)	Beckman Coulter	IBMULK1_TCRPGD
CD158b1,b2,j-154Eu (1:100)	Beckman Coulter	C65928
CD158a,h-163Dy (1:100)	Beckman Coulter	C65928
NKG2A-165Ho (1:400)	Miltenyi	130-122-329
NKp30-169Tm (1:50)	Beckman Coulter	C65929
CD33-170Er (1:400)	BioLegend	BLE3034419
EOMES-149Sm (1:400)	BD Biosciences	624084-EOMES
BCL-2-150Nd (1:800)	BD Biosciences	624084-BCL2
BCL-XL-158Gd (1:100)	Cell Signaling Technology	2764BF

Chemicals, peptides, and recombinant proteins

Human BD Fc Block™	BD Biosciences	Cat#564219
Lymphocyte separation medium	Eurobio	Cat#CMSMSL01-01
Cell-ID™ Intercalator-Ir_125 μM	Fluidigm	Cat#201192A
Cell-ID™ Intercalator-Rh_500 μM	Fluidigm	Cat#201103A
EQ™ Four Element Calibration Beads	Fluidigm	Cat#201078
Maxpar® Cell Staining Buffer	Fluidigm	Cat#201068
Maxpar® PBS	Fluidigm	Cat#201058
Fetal Bovine Serum, qualified	Gibco	Cat#10270-106
RPMI 1640 Medium	Gibco	Cat#11875093
Dimethyl Sulfoxide	Sigma-Aldrich	Cat#D4540
eBioscience™ Fixation/Transcription Factor Staining Buffer Set (Fixation/Permeabilization Solution and Perm/wash™ Buffer)	Thermo Fisher Scientific	Cat#00-5523-00
Bond-Breaker™ TCEP solution, neutral pH	Thermo Fisher Scientific	Cat#77720
Pierce™ Universal Nuclease for Cell Lysis	Thermo Fisher Scientific	Cat#88701
16% formic aldehyde (w/v) Pierce™, without methanol	Thermo Fisher Scientific	Cat#28906

Software and algorithms

CYTOF Software V6.0	Fluidigm	https://www.fluidigm.com/products-services/software https://www.fluidigm.com/products-services/software
OMIQ		https://www.omiq.ai/
FlowJo V10.6	Becton, Dickinson and Company	https://www.flowjo.com/solutions/flowjo/downloads
Cytosplore V2.3.1		https://www.cytosplore.org/

Other

115 Indium	Trace Sciences	Cat#115 _n
Maxpar® X8 Antibody Labeling Kit, 141Pr	Fluidigm	Cat#201141A
Maxpar® X8 Antibody Labeling Kit, 147Sm	Fluidigm	Cat#201147A
Maxpar® X8 Antibody Labeling Kit, 149Sm	Fluidigm	Cat#201149A
Maxpar® X8 Antibody Labeling Kit, 153Eu	Fluidigm	Cat#201153A
Maxpar® X8 Antibody Labeling Kit, 154Eu	Fluidigm	Cat#201154A
Maxpar® X8 Antibody Labeling Kit, 158Gd	Fluidigm	Cat#201158A
Maxpar® X8 Antibody Labeling Kit, 159Tb	Fluidigm	Cat#201159A
Maxpar® X8 Antibody Labeling Kit, 160Gd	Fluidigm	Cat#201160A
Maxpar® X8 Antibody Labeling Kit, 165Ho	Fluidigm	Cat#201165A
Maxpar® X8 Antibody Labeling Kit, 169Tm	Fluidigm	Cat#201169A
Maxpar® X8 Antibody Labeling Kit, 170Er	Fluidigm	Cat#201170A
Maxpar® X8 Antibody Labeling Kit, 174Yb	Fluidigm	Cat#201174A
Sterile syringe filter 0.2 μm	Dutscher	Cat#146560
Sterile syringe filter 0.45 μm	Dutscher	Cat#146561
Sterile syringe filter 0.8 μm	Dutscher	Cat#146562
Falcon® 5 mL Round Bottom Polystyrene Tube with Cell Strainer Snap Cap 35 μm	Dutscher	Cat#352063
96-well Clear V-Bottom Polypropylene Storage Plates	Corning Inc.	Cat#353263

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
15 mL high clarity polypropylene conical tubes	Corning Inc.	Cat#352096
50 mL high clarity polypropylene conical tubes	Corning Inc.	Cat#352070
5 mL round bottom polypropylene tubes	Dutscher	Cat#351235
Pre-separation filters (30 µm)	Miltenyi	Cat#130-041-407
Amicon® Ultra-0.5 mL Centrifugal Filters Ultra-0.5-3K	Millipore	Cat#UFC500396
Amicon® Ultra-0.5 mL Centrifugal Filters Ultra-0.5-50K	Millipore	Cat#UFC505096
Micro tube 1.5 mL	Sarstedt	Cat#72-690
Megafuge 2.0R	Heraeus Instruments	NA
Biofuge fresco	Heraeus Instruments	NA
Eppendorf 5810R	Eppendorf Corporate	NA
Microscope Olympus CKx31	Olympus Life Science	NA
Glasstic slide 10 with grids	Kova International	NA
Vortex1 genie touch mixer	Scientific Industries	NA
Chemical hood Astec sensair	Astec	NA
A set of pipettes (2.5/10/200/1,000 µL)	Eppendorf Corporate	NA
Pipet.aid	Drummond Scientific corporation	NA
MilliQ 1 x pure water system	Merck	NA
Nanodrop 1000 spectrophotometer	Thermo Fisher Scientific	NA

* this antibody was produced by the immunomonitoring department of the IPC.

MATERIALS AND EQUIPMENT

RPMI 10% FCS	Volume (mL)
RPMI 1640 Medium	450
FCS (heat inactivated and filtered)	50
Can be stored at 4°C for 1 month.	

RPMI 2% FCS	Volume (mL)
RPMI 1640 Medium	490
FCS (heat inactivated and filtered)	10
Can be stored at 4°C for 1 month.	

Pierce universal nuclease	Volume (µL)
RPMI 1640 2% FCS	10 000
Pierce Universal Nuclease	1
This solution is prepared extemporaneously.	

Cisplatin	Volume (µL)
RPMI 1640 0% FCS	5 000
Cisplatin 5 mM	1
This solution is prepared extemporaneously.	

Formaldehyde 2%	Volume (µL)
Formaldehyde 16%	125
Maxpar PBS	875
This solution is prepared extemporaneously.	

Perm wash	Volume (μL)
MilliQ water	9 000
Perm buffer 10x	1 000

This solution is prepared extemporaneously.

Freezing media	Volume (μL)
FCS	90
DMSO	10

This solution is prepared extemporaneously.

△ **CRITICAL:** Pierce Universal Nuclease (final concentration=2.5 U/mL) and Cisplatin (final concentration=1 μM) must be prepared extemporaneously.

△ **CRITICAL:** In order to avoid contamination with elements likely to damage the detector of the mass cytometer, glass, material that could have been exposed to soap, and autoclaved material cannot be used. The use of polystyrene tubes or plates and unfiltered tips is prohibited as well.

STEP-BY-STEP METHOD DETAILS

⌚ Timing: 5 h

This section describes a workflow for staining samples with 28-metal isotope panel in [key resources table](#). Unless otherwise stated, cell pellets are suspended by pipetting.

PBMC sample thawing

⌚ Timing: 2 h

This step details how to thaw cryopreserved PBMCs before staining.

1. Prepare 15 mL conical tubes with RPMI 10% Fetal Calf Serum (FCS).
2. Thaw each sample vial in a 37°C water bath until a small pellet of ice appears.
3. Transfer the contents of PBMC vial to a 15 mL conical tube and rinse the vial.
4. Centrifuge the cells at 400 \times g for 5 min at 20°C–22°C.
5. Discard flow-through and resuspend the pellet in 10 mL of RPMI 2% FCS.
6. Add 1 μL of Pierce Universal Nuclease 25 kU and incubate at 37°C for 30 min.
7. Filter the cells with a 30 μm filter and proceed to cell count.
 - a. Mix 10 μL of cell suspension with 10 μL of trypan blue.
 - b. Load this mix onto KOVA slide and count cells.
8. Centrifuge all samples: 400 \times g, 5 min, 20°C–22°C.
9. Resuspend the pellet in CSM, and distribute 3.10⁶ cells/well up to 250 μL in 96-well polypropylene plates.

Viability stain

⌚ Timing: 10 min

This step details how to label dead cells.

10. Centrifuge the plate: $1,017 \times g$, 3 min, 20°C – 22°C .
11. Prepare cisplatin working solution.
12. Add $100 \mu\text{L}$ / well of cisplatin diluted at final a concentration of $1 \mu\text{M}$ and incubate samples for 5 min at 37°C .

Note: Viability stain can be performed using the same procedure replacing cisplatin by 103Rh. Use 103Rh at a dilution of 1:500 in RPMI and incubate 15 min at 20°C – 22°C .

13. Add $100 \mu\text{L}$ of RPMI to stop the reaction and centrifuge the plate $1,017 \times g$, 3 min, 20°C – 22°C .
14. Aspirate the flow-through and wash the cells with $200 \mu\text{L}$ of CSM, centrifuge $1,017 \times g$, 3 min, 20°C – 22°C .

Figure 2.

⚠ CRITICAL: Since cisplatin is a DNA alkylating agent, steps 11–14 should be performed under a chemical hood.

Blocking Fc receptors and extracellular staining

⌚ Timing: 1 h 15 min

This step details how to perform FcR blocking, extracellular staining and cell fixation.

15. Prepare FcR blocking reagent by diluting Fc Block 1:10 with CSM.

Note: FcR blocking can be replaced by CSM supplemented with 10% FCS (Figure 4).

16. Add $100 \mu\text{L}$ of diluted FcR blocking reagent to each well, mix by pipetting and incubate for 10 min at 4°C .
17. Add $100 \mu\text{L}$ of CSM and centrifuge $1,017 \times g$, 3 min, 4°C .
18. Add $25 \mu\text{L}$ of extracellular master mix per sample and incubate for 1 h at 4°C .

Note: Extracellular mix is stable at -80°C for one year (data not shown).

19. Add $175 \mu\text{L}$ of CSM and centrifuge $1,017 \times g$, 3 min, 4°C .
20. Aspirate the flow-through and wash the cells with $200 \mu\text{L}$ of CSM, centrifuge $1,017 \times g$, 3 min, 4°C .

Note: A second incubation for secondary antibodies can be added before 2% formaldehyde fixation step.

21. Aspirate the flow-through and add $100 \mu\text{L}$ of formaldehyde 2% and incubate for 15 min at 20°C – 22°C .
22. Add $100 \mu\text{L}$ of CSM and centrifuge $1,017 \times g$, 3 min, 4°C .

Note: This procedure can be stopped at this stage and cells stored in CSM for 72 h at 4°C . After 72 h, the intracellular labeling can be processed (Figure 3).

Intracellular staining

⌚ Timing: 1 h

This step details how to perform the permeabilization in order to stain the intracellular and intranuclear markers.

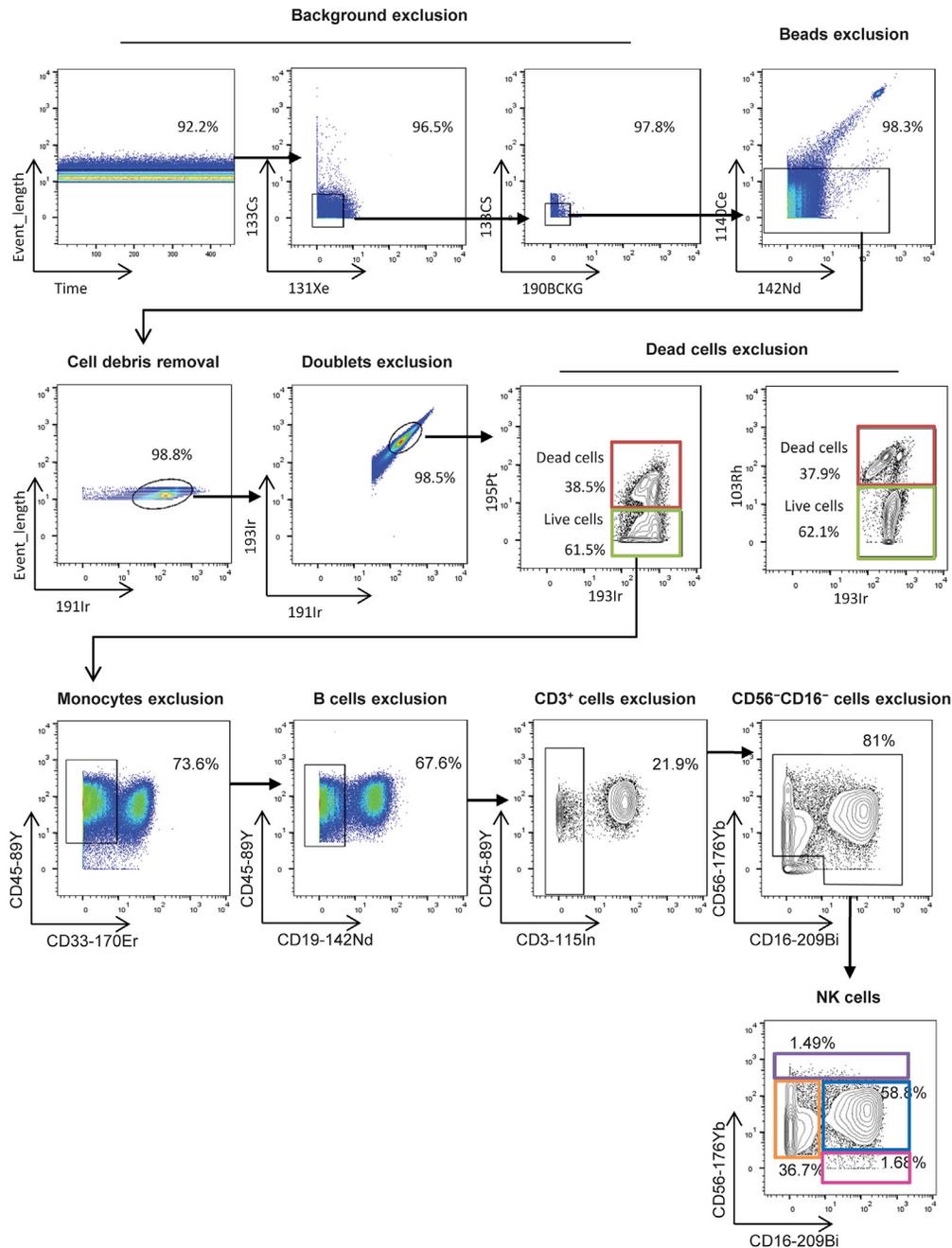


Figure 2. Gating strategy

PBMCs from healthy volunteers were stained with metal-labeled antibodies and analyzed by mass cytometry (CyTOF Helios®). After removal of beads, cells were pre-gated as DNA⁺. Dead cells were excluded based on CisPt or Rh positivity. Monocytes were excluded based on CD33 expression. B cells and T cells were excluded based on CD19 and CD3 expression, respectively. Four subsets of NK cells were defined based on CD56 and CD16 expression: CD56^{bright} NK cells, CD56^{dim}CD16⁺ NK cells, CD56^{dim}CD16⁻ NK cells, and unconventional CD56⁺CD16⁺ NK cells.

23. Permeabilize using 200 μ L per well of Foxp3 Staining Buffer Set by diluting Fixation/Permeabilization concentrate in Perm Diluent (1/4 dilution).
24. Add 25 μ L of intracellular master mix per sample and incubate for 30 min at 4°C.
25. Add 175 μ L of Perm/Wash working solution and centrifuge 1,017 \times g, 3 min, 4°C.

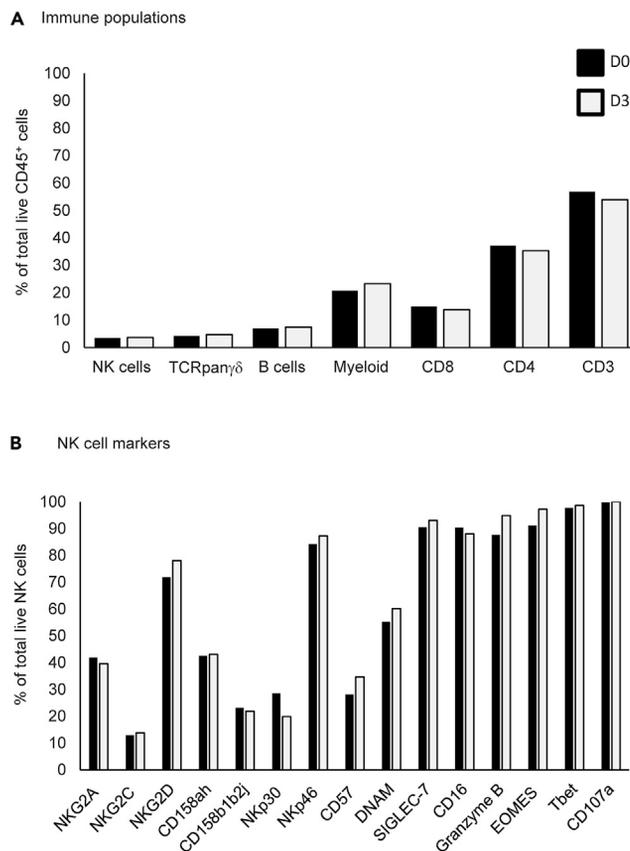


Figure 3. Impact of the staining procedure on NK cell phenotyping

The labeling procedure including extracellular and intracellular staining was performed from a control sample either on day 0, or splitted into extracellular staining on day 0, and intracellular staining on day 3.

(A) Displays the frequency of immune populations with each of the staining procedures.

(B) Displays the frequency of NK cell markers with each of the staining procedures.

- Aspirate the flow-through and wash the cells with 200 μ L of 1 \times Perm/Wash, centrifuge 1,017 \times g, 3 min, 4°C.

Fixation and DNA intercalator staining

⌚ Timing: 10 min

Finally, samples are fixed with DNA intercalator to identify cells after acquisition.

- Prepare extemporaneously 2% formaldehyde solution by diluting fresh 16% formaldehyde with PBS.
- Add 100 μ L of 2% PFA + DNA intercalator (Cell-ID™ Intercalator-Ir) diluted at 1:1,000 per sample and incubate 12–16 h.

Note: At this stage, labeled samples can be frozen or directly acquired on the Helios system.

Sample freezing

⌚ Timing: 30 min

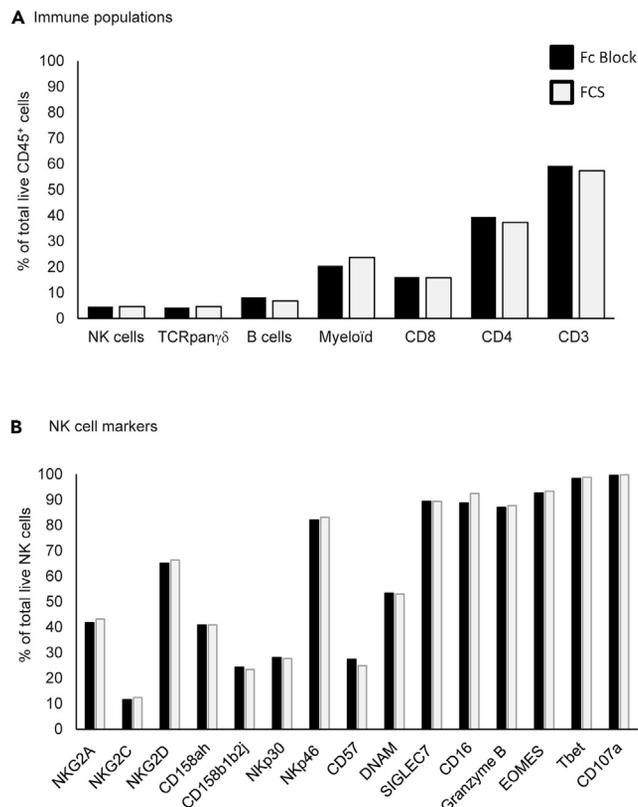


Figure 4. Impact of the blocking procedure on NK cell phenotyping

Intracellular and extracellular aspecific epitopes from a control sample were blocked using PBS 0.5 mg/mL Human Fc Block, or PBS 10% FCS.

(A) Displays the frequency of immune populations with each of the blocking procedures.

(B) Displays the frequency of NK cell markers with each of the blocking procedures.

Here, we propose a freezing strategy that enables labeling stability for a maximum storage period of one week.

29. Wash the cells with 100 μ L of CSM and centrifuge 1,017 \times g, 3 min, 4°C.
30. Aspirate the flow-through and wash the cells with 200 μ L of CSM and centrifuge 1,017 \times g, 3 min, 4°C.
31. Add 100 μ L of freezing media per sample and store directly in microtubes at -80°C until the acquisition.

Note: Labeled samples can be stored at -80°C freezer for one week without losing staining quality.

Instrument tuning and sample acquisition

⌚ Timing: 45 min for 1 M cells

The startup procedure follows the instrument manufacturer's guidelines involving checking the instrument performance, monitoring background and tuning the system.

32. Thaw samples at 20°C–22°C, transfer the cells into a 96-well plate, and add 100 μ L of PBS.
33. Centrifuge samples for 1,017 \times g, 3 min, 4°C.

34. Aspirate flow-through by pipette.
35. Repeat steps 32–34.
36. Add 200 μ L of deionized water (DIW) and proceed to cell count.

Note: Wash the samples in DIW only after instrument start-up and tuning validation because samples should not remain in DIW for more than 1 h. Thus, samples must be thawed gradually.

37. Centrifuge samples for $1,017 \times g$, 3 min, 4°C and aspirate the flow-through.
38. Prepare the stock solution of 1:10 EQ Bead/DIW.

Note: Depending on the sample (cell line, tumor...) a higher dilution in EQ Beads/DIW is necessary to avoid fluidic line clogging.

39. Aspirate flow-through by pipette and resuspend one sample with EQ Bead/DIW by adjusting concentration to achieve an acquisition rate of 500 events/second i.e., 1 M/mL.
40. Normalized and concatenated the files before analysis.
41. Several algorithms can be used to for data analysis.

Note: For analysis of immune populations that represent at least 1% of the total PBMC, a minimum of 50 000 cells should be acquired. However, when possible, we recommend the acquisition of a minimum of 500 000 cells in order to enable the analysis of rare immune subsets, or subsets that are under-represented in specific pathologic conditions such as hematologic malignancies, in which circulating tumor cells may represent up to 99% of the total PBMC.

EXPECTED OUTCOMES

In this section, we describe a gating strategy to identify NK cells in peripheral blood from healthy donor (Figure 2). Raw data (.FCS) were obtained after staining and acquisition then normalized using Fluidigm Software (.fcs). These data were analyzed using FlowJo Software to remove beads, clog and dead cells. On CD45⁺ live cells we exclude CD33⁺ and CD19⁺ cells. Based on CD3, CD56 and CD16 expression, we identified subpopulations of NK cells.

Unsupervised clustering using t-SNE also enables to visualize the main immune populations obtained from our staining protocol: myeloid cells, NK cells, B cells, T cells, $\delta\gamma$ T cells (Figure 5). We focused on NK cells to refine their phenotype. Manually pre-gated total NK cells were exported; selected markers CD56, NKG2A, CD158b1b2j, CD158ah, CD57, NKG2C, CD16 were used to identify seven clusters of NK cells. These clusters illustrate the step of NK cell maturation from CD56^{bright} to memory-like cells, as well as CD56⁻ CD16⁺ unconventional NK cells (Figure 6).

LIMITATIONS

We propose a validated staining procedure with 28 markers and 5 channels used for viability and DNA identification, so we can use free channel to add more markers. Be careful, depending on cells and markers, we can lose the specific staining comparing to single labeling. It can be a steric housing problem.

This problem can be resolved using successive incubations. Antibodies targeting weakly represented antigens on the cell surface can be incubated before antibodies targeting strongly represented antigens on the cell surface. This allows overcoming steric housing problem.

TROUBLESHOOTING

Problem 1

Batch effect.

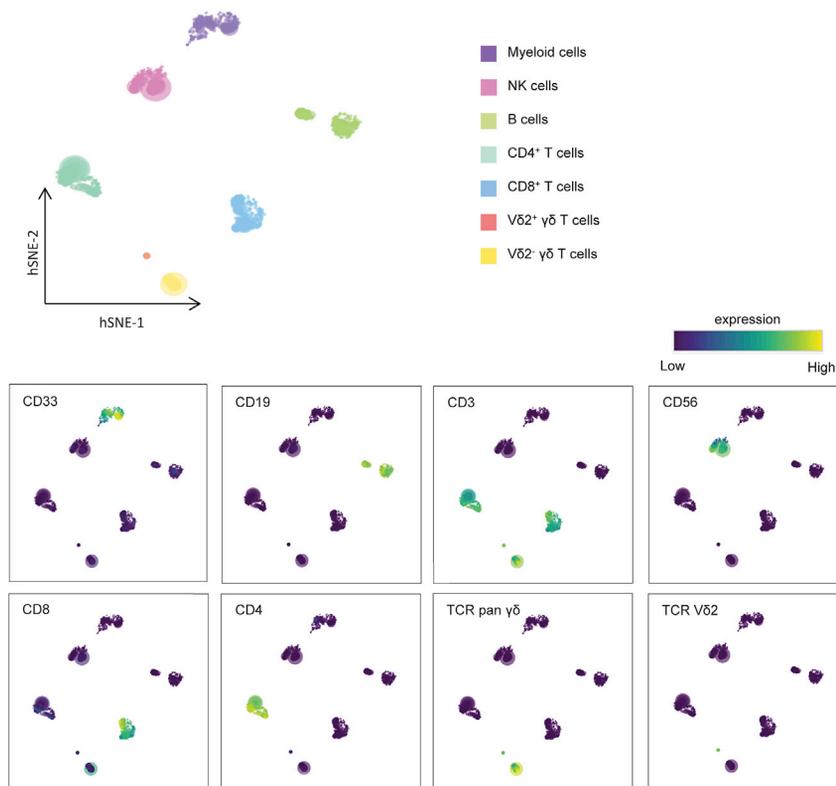


Figure 5. High dimensional analysis of PBMCs

Consensus files of PBMCs from 22 healthy volunteers were generated with fixed number of cells and exported in Cytosplore for h-SNE analysis. h-SNE enables identification of myeloid cells (CD33⁺), B cells (CD33⁺, CD19⁺), NK cells (CD33⁺ CD3⁻ CD56^{+/+}), CD8⁺ T cells (CD33⁺ CD3⁺ CD8⁺), CD4⁺ T cells (CD33⁺ CD3⁺ CD4⁺), Vδ2⁺ γδ T cells (CD33⁺ CD3⁺ Vδ2⁺), and Vδ2⁻ γδ T cells (CD33⁺ CD3⁺ Vδ2⁻). The following settings were used for hSNE analyses: number of scales: 3; #RW for Monte Carlo: 15; Random walks threshold: 1.50; Random walks length: 15; Filter percentile: 0.50.

Several factors can be at the origin of batch effect such as inter-operator variability, instrument variability as well as duration of acquisition.

Potential solution

Several algorithms are available to correct the batch effect (Lo et al., 2022; Pedersen et al., 2022; Van Gassen et al., 2020). To do this, the use of validated and appropriate internal control is required. Important: this control must express all the markers included in the panel.

Problem 2

Antibodies conjugation to metal isotopes failed.

Potential solution

To ensure optimal metal conjugation, it is recommended to use purified antibodies that respect the following rules:

- Antibody type: monoclonal.
- Antibody class: IgG.
- Buffer: BSA- and glycerol-free.

Alternatively, it is possible to use flow cytometry antibodies labeled with a fluorochrome and to use an antibody targeting the fluorochrome labeled with a metal.

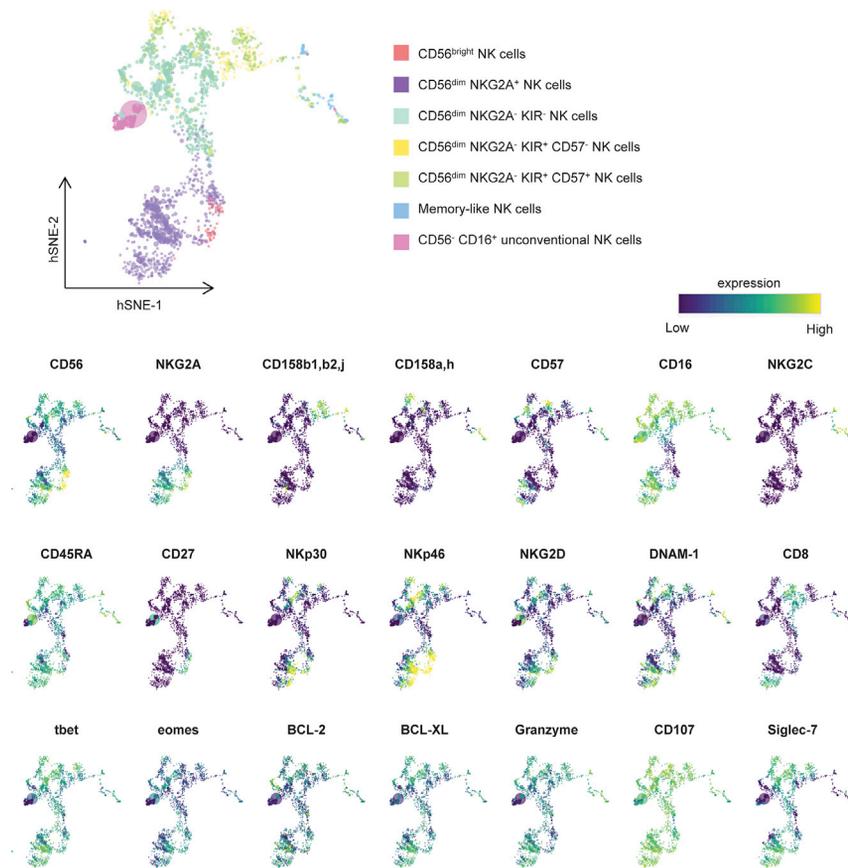


Figure 6. High dimensional analysis of peripheral NK cells

Total NK cells from peripheral blood were manually pre-gated. Consensus files were generated from 22 healthy volunteers with fixed number of NK cells and exported in Cytosplore for h-SNE analysis. h-SNE enables visualization of 7 clusters of NK cells based on CD56, NKG2A, CD158a,h,CD158b1,b2,j, CD57, NKG2C and CD16 expression. The following settings were used for hSNE analyses: number of scales: 3; #RW for Monte Carlo: 15; Random walks threshold: 1.50; Random walks length: 15; Filter percentile: 0.50.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Anne-Sophie Chretien (anne-sophie.chretien@inserm.fr).

Materials availability

This study did not generate new unique reagents.

Data and code availability

There is no dataset/code associated with the present article.

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AUTHOR CONTRIBUTIONS

Conceptualization: J.A.N., N.V., H.L., E.G., D.O., and A.S.C.; methodology: H.L. and E.G.; validation: H.L., E.G., D.O., and A.S.C.; formal analysis: A.B.A., M.S.R., J.W., and A.S.C.; investigation: A.B.A., M.S.R., S.F., E.G., and N.B.; resources: H.L. and N.V.; data curation: J.W. and A.S.C.; writing – original draft: A.B.A., M.S.R., and L.G.; writing – review & editing: L.G. and A.S.C.; visualization: A.B.A., M.S.R., P.L.B., L.G., and A.S.C.; supervision: H.L., D.O., and A.S.C.; project administration: H.L., D.O., and A.S.C.; funding acquisition: J.A.N., N.V., D.O., and A.S.C.

DECLARATION OF INTERESTS

E.G. is currently employed by Fluidigm. D.O. is a cofounder and shareholder of Imcheck Therapeutics, Alderaan Biotechnology, Emergence Therapeutics, and Stealth IO.

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