



Flow cytometry technique for identification and functional analysis of NK cells

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Abstract

Introduction: Natural killer (NK) cells are lymphocytes subset that contribute to innate immunity through their cytotoxic function. This cytotoxic function is generally provided by degranulation of CD56^{dim}CD16^{pos} NK subpopulation during various infections. However, the identification of NK cells can be complex due to their different functions. Here we propose a protocol for the identification of CD56^{dim}CD16^{pos} NK cells by flow cytometry.

Methods: Peripheral blood mononuclear cells (PBMCs) from healthy individuals were isolated by density gradient centrifugation using Ficoll-Paque; then NK cell subsets and degranulation markers were characterised by flow cytometry using Annexin-V, CD3, CD56, CD16 and CD107a staining.

Results: After showing the gating strategies for the identification of unstained and fluorescence minus one (FMO) control, the different NK cell subpopulations will be displayed: CD56^{neg}CD16^{pos}, CD56^{dim}CD16^{pos}, CD56^{bright}CD16^{dim}, CD56^{dim}CD16^{neg} and CD56^{bright}CD16^{neg}. Finally, the gating strategy for the functional analysis of the degranulation of the CD56^{dim}CD16^{pos} subpopulation will be presented: CD56^{dim}CD16^{pos}CD107a^{pos}.

Conclusion: This protocol will facilitate the functional analysis and identification of degranulated NK cells during various infections.

Keywords: Natural killer (NK) cell subsets, Peripheral blood mononuclear cells, Degranulation, Flow cytometry

Introduction

Natural killer (NK) cells are granular subset of lymphocytes that are part of the innate immune system. They play a crucial role in the initial defence against viral, bacterial or parasitic infections (1). In humans, NK cells are CD3 negative cells and constitute about 5-15% of peripheral blood mononuclear cells (PBMCs), and they can typically be identified by the expression of the surface markers CD56 and CD16 (2, 3). There are two main subsets of NK cells with distinct maturation and functional properties (4). CD56^{bright}CD16^{neg} subsets are efficient producers of pro-inflammatory cytokines and represent 10% of the peripheral NK cells (5). In contrast, CD56^{dim}CD16^{pos} NK cells represent about 90% of peripheral NK cells in humans and

are known for their high cytotoxic effector properties (6). Some reports have described a subset of CD56^{neg}CD16^{pos} NK cells that are expanded in the context of chronic viral infections such as Human Immunodeficiency Virus (HIV) patients, (7) or during Hepatitis C Virus (HCV) infections (8). Regarding the cytolytic function of NK cells, the most abundant protein in the cytotoxic granules is CD107a, and its expression on the surface is considered as a marker of degranulation (9). Due to the important role that NK cells play in immunity, and the emerging exploration of the NK cell platform for immunotherapy in different diseases, analysis of NK cell responses is becoming a useful tool for assessing a variety of clinical conditions, such as cancer, post-stem cell transplantation to assess risk of infection and diagnosis of immunodeficiency syndromes. However, the identification of NK cells can be complex due

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to their different functions. Here we propose a simple and easy method for identifying the different subpopulations of NK cells and analysing their cytotoxic function by flow cytometry.

Methods

Materials

All materials used in this protocol are listed in the Table 1.

Buffer and media preparation

Fetal calf serum preparation

Inactivate Fetal Calf Serum (FCS) complement with humid heat for 30 minutes at 56°C before use.

RPMI 1640 Complete Medium preparation

To 500mL RPMI 1640, add 10% inactivated FCS supplemented with Gentamycin at a concentration of 50µg/mL, Penicillin/Streptomycin at a concentration of 100µg/mL and L-glutamine at a concentration of 2 mM/mL.

Cell Cryopreservation Medium (Freezing medium)

Add 20% DMSO to the inactivated FCS to obtain the freezing medium. Then chill the medium for 5 to 10 minutes to limit the exothermic reaction that occurs when DMSO is added to FCS, before adding it to the cells.

FACS buffer preparation

To sterile PBS add 2% of inactivated FCS to obtain the FACS Buffer.

Peripheral Blood Mononuclear Cells (PBMCs) Isolation

PBMCs from 3 healthy donors were isolated using the Ficoll-Paque density gradient centrifugation method.

1. Collect 20mL of blood by vein-puncture in EDTA tubes from each participant as followed:
2. Add 10 mL of Ficoll-Paque to three conical 50mL tube.
3. Dilute the whole blood at 1:2 with sterile PBS in a 50mL conical tube and mix gently. In this protocol we have used 10mL of PBS (1x) and 20mL of whole blood.
4. Carefully transfer the diluted blood sample on the top of Ficoll-Paque into each 50mL conical tube avoid mixing the Ficoll-Paque and the diluted blood.
5. Centrifuge tubes at 2000×g for 20 minutes at room temperature.
6. Collect the white interphase layer of PBMCs with a sterile pipette in a 50mL conical and wash twice by centrifugation at 1300 rpm for 8 minutes with 10 mL RPMI 1640 medium previously prepared.
7. Resuspend the cells pellet in 1mL of complete medium.
8. Count viable cells by Trypan Blue exclusion technique.
9. If needed, add the cells suspension with RPMI 1640 medium to obtain 1×10^5 cells/mL.

NB: The entire isolation procedure must be carried out under a hood and the cells must be kept on ice.

PBMCs cryopreservation

The rest of the fresh cells can be preserved for further use.

1. Prepare cell cryopreservation medium.
2. We usually freeze 1×10^5 cells per cryovial.
3. Aliquot into 2mL cryotubes a total volume of 500 µL of cells each containing 500 µL of freezing medium to obtain a final volume of 1mL.
4. Place all the vials in a freezing container and store it at -80°C . Then, transfer the vials to an appropriate container and store them in liquid nitrogen.

Note: The cryopreservation process needs to be fast. As it affects cell viability, try to shorten the time that cells are exposed to DMSO before putting them in the freezer.

Immunophenotyping of NK cells

The surface staining and fixation procedures are described in detail in this step.

Use fresh PBMCs for phenotypic and functional assays. To assess NK cells degranulation, a surface staining can be performed.

1. Prepare the tubes for NK cells immunophenotyping as followed:
 - a. Unstained tube: negative control.
 - b. Fluorescence minus One (FMO) tubes: FMO control contains all the fluorochromes in a panel, except for the one that is being measured.
 - c. Tube test: it contains all the fluorochromes in a panel.
2. Pipette 100µL PBMCs suspension containing 1×10^5 cells in each tube.
3. Wash the cells, by centrifugation at 1500 rpm for 5 minutes with 200µL of FACS buffer and discard the supernatant.
4. Stain the cell pellets in the dark with appropriate markers in appropriate tube, following the manufacturer's instructions. In this protocol we used anti-hCD3-APCA750; anti-hCD16-FITC and anti-hCD56-PE as specific markers of NK cells; anti-h Annexin-V PB450A for viability staining; anti-hCD107a APC for degranulation assays.
5. Vortex the stained cells and incubate under dark for 30 minutes at 4°C .
6. Repeat the step 3 and resuspend the cells in 100 µL of FACS buffer for sample acquisition on the flow cytometer.

Note: Alternatively, you can also prepare one control per marker that you want to determine (Single stained) to assess the effectiveness of markers.

Compensation on flow cytometer

In this protocol we used VersaComp Antibody Capture Bead Kit to correct spectral overlap by performing fluorescence compensation following the manufacturer's instruction.

Note: Compensation can be also done after sample acquisition with different flow cytometry data analysis software (e.g., FlowLogic, FlowJo, etc.).

Quantification and Statistical Analysis

To assess the effector functions of human CD56^{dim}CD16^{bright} NK cells, we measured degranulation. FlowJo software version 10.10.0 was used for the analysis of “fcs” files of flow cytometry data.

Expected results

Identification of controls

In this protocol, we developed a control strategy for the correct identification of human CD56^{dim}CD16^{bright} NK cells

in peripheral blood and the analysis of the degranulation function of this subpopulation by flow cytometry.

Firstly, we started gating the flow data by using the Time gate based on the size (SSC: side scatter) and Time to identify acquisition issues (Figure 1A). Then we created lymphocytes gate based on their granularity (FSC: forward scatter) and size (SSC) (Figure 1B), and individual cells were selected based on FSC-A (area) and FSC-H (height) (Figure 1C). Following these individual cells, the unstained control (Figure 1D) and the FMO controls (Figure 1E-I) were determined, by selecting the fluorochrome of the marker to be measured according to lateral scattering (SSC). For a successful FMO control the percentage of cells counted should be < 1%.

Table1: Key materials and equipments

REAGENT or RESOURCE	SOURCE
Antibodies	
Anti-human CD56-PE (clone MEM-188)	Biolegend, Koblenz, Germany
Anti-human CD16-FITC (clone 3G8)	Biolegend, Koblenz, Germany
Anti-human CD3-APC A-750 (clone UCHT1)	Biolegend, Koblenz, Germany
Anti-human CD107a APC (clone H4A3)	Biolegend, Koblenz, Germany
Annexin-V PB450A (lot: B380332)	Biolegend, Koblenz, Germany
Biological Samples	
Peripheral blood mononuclear cells (PBMCs) from healthy donors	Volunteers recruited at the National Blood Transfusion Centre (Centre National de transfusion sanguine,CNTS)
Reagents	
EDTA tubes of 10 mL	BD Vacutainer, K2E 18.0 mg, BD, Plymouth, UK
Ficoll-Paque Plus	Greiner Bio-One, GmbH, Germany
Fetal Calf Serum (FCS)	PAN Biotech, Aidenbach, Germany
Dimethylsulfoxide (DMSO)	Sigma Aldrich, St Louis, USA
Gentamycin	Life Technologies Corporation Grand Island, USA
RPMI 1640 medium	Gibco, California, USA
L-glutamine	Life Technologies Limited, Paisley, UK
Penicillin-Streptomycin	Life Technologies Corporation Grand Island, USA
Phosphate Buffered Saline (PBS)	Gibco, Thermo Fisher Scientific
Trypan Blue Solution	Life Technologies Corporation, Grand Island, USA
VersaComp Antibody Capture Bead Kit	Beckman Coulter, Brea, California, USA
Software and Algorithms	
FlowJo™ Version 10.10.0	FlowJo LLC
GraphPad Prism v9.0	Prism-GraphPad
Other (Equipement, consummables)	
Analyzer flow cytometer (Cytoflex)	Beckman Coulter Technology, Suzhou, China
Falcon® conical 50 mL centrifuge tubes	Falcon Greiner Bio one, Frickenhausen, Germany
Falcon conical 15 mL Centrifuge Tubes	Falcon Greiner Bio one, Frickenhausen, Germany

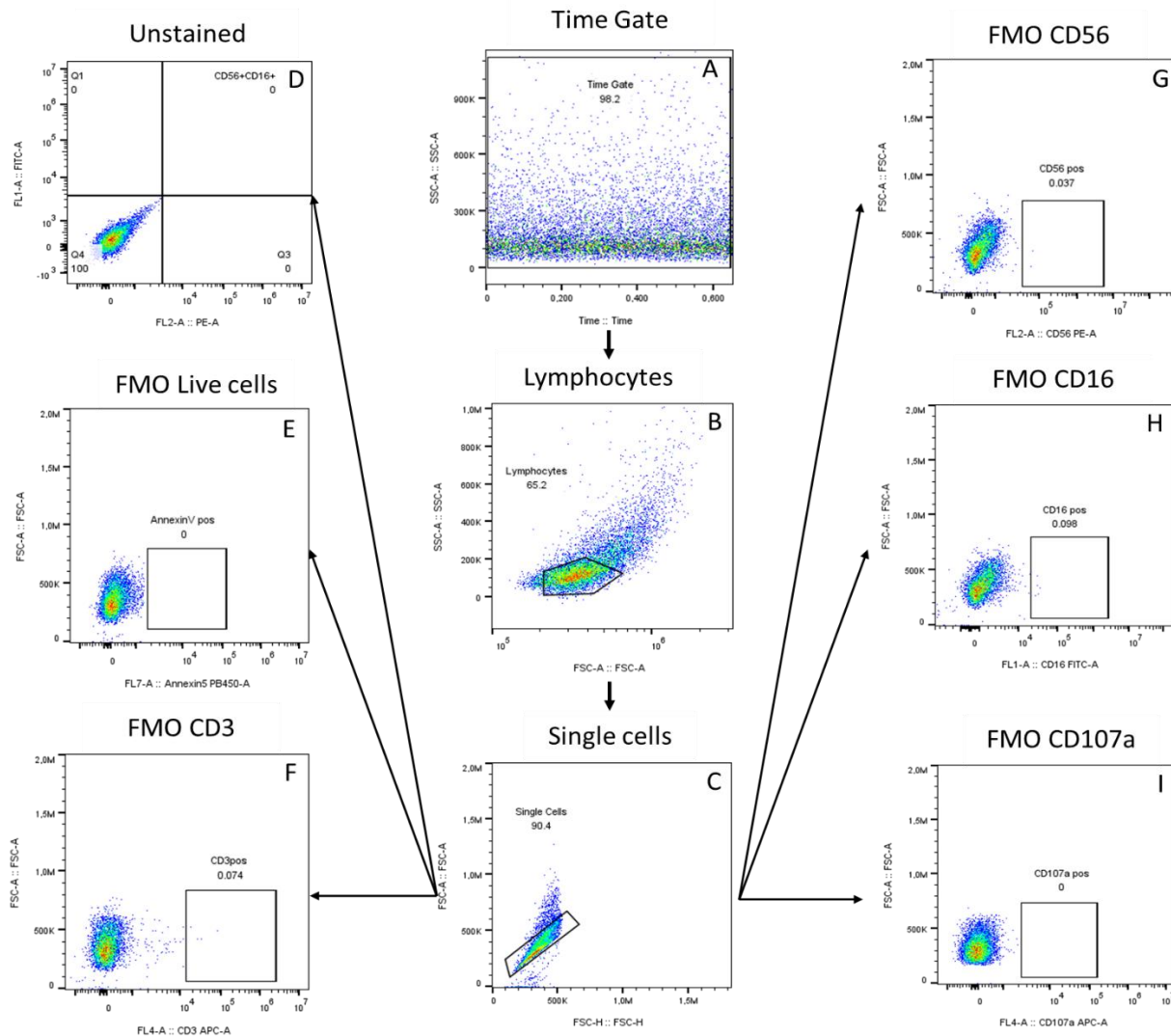


Figure 1: FMO Controls gating strategy. The arrows indicate the gating of controls A) Identification of acquisition issues in the Time gate based on the SSC-A surface area and Time B) Selection of lymphocytes cells based on FSC (granularity) and SSC (size); C) Single cells based on FSC-A (area) and FSC-H (height); D) Unstained; E) FMO Annexin-V; F) FMO CD3; G) FMO CD56; H) FMO CD16; I) FMO CD107a.

Note 1: The time gate may have debris in the data due to air or obstructions during acquisition. In this case it is recommended to create the time gate by eliminating these problematic areas.

Note 2: A second purification of individual cells can also be performed by selecting individual cells according to SSC-A surface area and SSC-H height by side-scattering.

Functional analysis of NK cells: degranulation assay

Once the controls were determined, CD56^{dim}CD16^{bright} NK cells were correctly identified, and the effector functions of this subset were assessed. We started gating the flow data by using the Time gate based on the size (SSC: side scatter) and Time to identify acquisition issues (Figure 2A). We created lymphocyte gates based on their forward scatter (FSC) and side scatter (SSC) parameters (Figure 2B). Then,

individual cells were selected based on FSC-A (area) and FSC-H (height) (Figure 2C). Then an exclusion channel (Annexin-V viability dye) was included in our control strategy to specifically study viable cells (Figure 2D). From the viable Annexin-V- (negative) cells anti-CD3 monoclonal antibody was included to exclude T cells (Figure 2E). Thus, the NK cells were identified for the non-T cells population, based on the expression of the surface markers CD56 and CD16 (Figure 2F) and different subsets were selected from

CD56^{pos} and CD16^{pos} (Figure 2G). To measure the degranulation of the CD56^{dim}CD16^{pos} NK cells subset, we included anti-CD107a monoclonal antibodies, and

degranulated NK cells expressing CD107a marker, were identified from CD56^{dim}CD16^{pos} (Figure 2H).

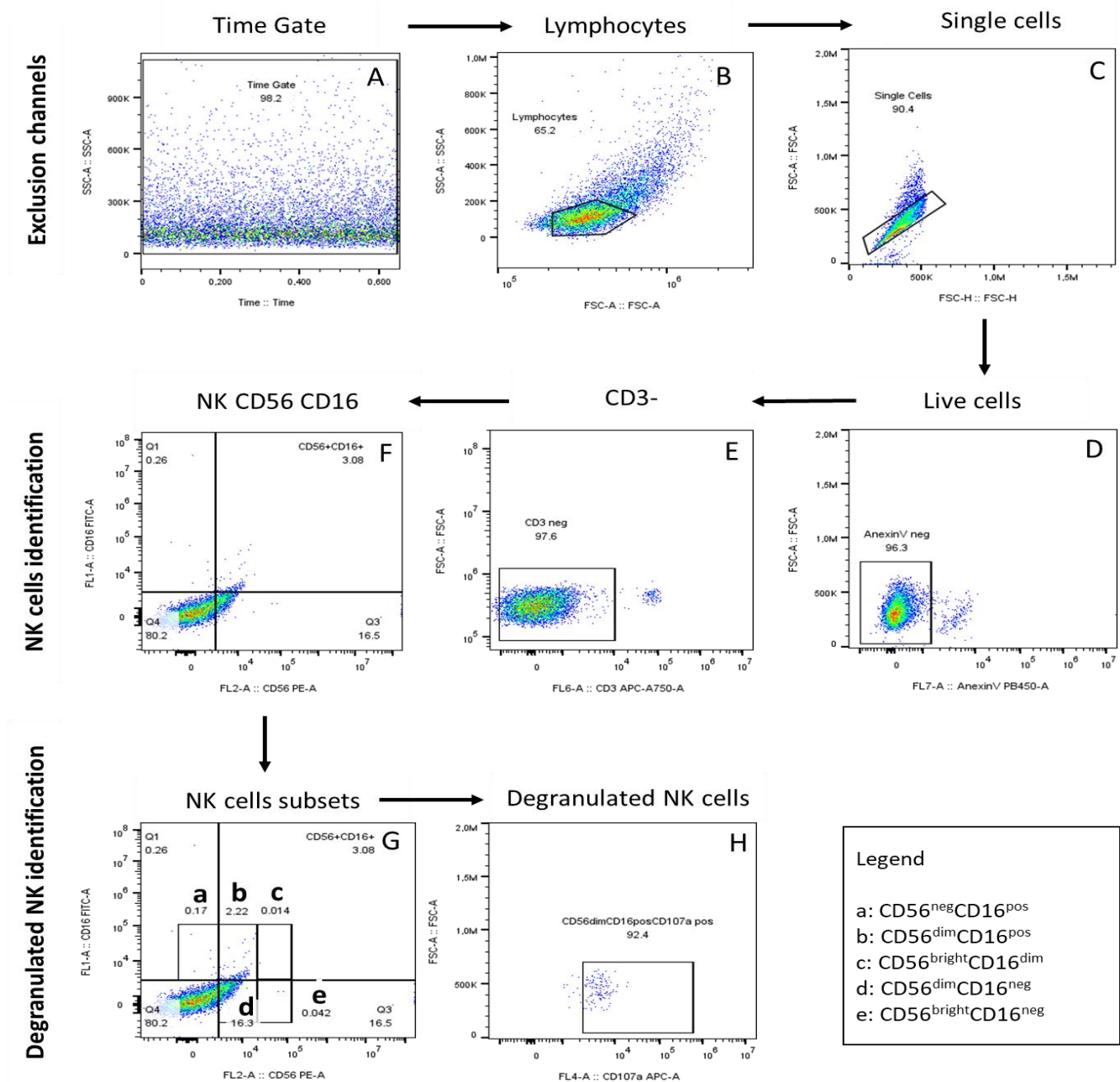


Figure 2: Gating strategy of degranulated NK cells identification. The arrows show the gating steps for the expression of CD107a on the NK cells. A) Identification of acquisition issues in the Time gate B) Selection of lymphocytes based on FSC-A and SSC-A; C) Single cells based on FSC-A and FSC-H forward scatter; D) Live cells E); CD3neg cells F); NK cells based on the CD56 and CD16 markers G); NK subsets cells **a)** CD56^{neg}CD16^{pos} **b)** CD56^{dim}CD16^{pos} **c)** CD56^{bright}CD16^{dim} **d)** CD56^{dim}CD16^{neg} **e)** CD56^{bright}CD16^{neg} H); Degranulated NK cells expressing CD107a identified from CD56^{dim}CD16^{pos} subset.

Limitations

NK cells play an important role in innate immune responses. In this protocol we describe a method to identify different NK cells subsets and their degranulation function. This protocol uses PBMCs for the identification of NK cells, and this identification included only anti- Annexin-V and anti-CD3 exclusion channel for the non-viable cells and T cells respectively, since NK cells are CD3- (negative) (10). Although T cells are excluded, some B cells may interfere with the identification of NK cells. However, to ensure the purity of NK cells we propose to include a B cell exclusion channel with anti-CD19 and anti-CD14 monoclonal antibodies. Furthermore, annexin-V is an apoptotic cells marker and cannot be used to identify other type of dead cells. We propose then to use a complete viability marker for the analysis of cell viability such as LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit.

The regulated expression of degranulated NK cells may be due to direct staining of NK cells after isolation. However, the effector function of NK cells underlies their activation through IL-2 or other stimuli (11). Thus, cells stimulation with Phorbol 12-myristate 13-acetate (PMA), IL-2+IL-12+IL-18 or antibodies for 24 or 48 hours, depending on the type of infection studied, would increase the percentage of degranulated cells. Nevertheless, the gating strategy proposed in this protocol enables NK cells and their degranulation functions to be properly identified.

Conflict of Interests

The authors declared no conflict of interest.

Author's contribution

Simfele Hombamane Christelle: Conceptualization, Formal Analysis, Writing Original Draft, Methodology; **Nadjir Koboyo Liza:** Enrolment; **Katawa Gnatoulma:** Conceptualization, Investigation, Data curation, Project Administration, Funding Acquisition, Methodology, Resources, Supervision; **Kolou Malewe:** Validation.

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