Video Article

Flow Cytometry-based Assay for the Monitoring of NK Cell Functions

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Abstract

Natural killer (NK) cells are an important part of the human tumor immune surveillance system. NK cells are able to distinguish between healthy and virus-infected or malignantly transformed cells due to a set of germline encoded inhibitory and activating receptors. Upon virus or tumor cell recognition a variety of different NK cell functions are initiated including cytotoxicity against the target cell as well as cytokine and chemokine production leading to the activation of other immune cells. It has been demonstrated that accurate NK cell functions are crucial for the treatment outcome of different virus infections and malignant diseases. Here a simple and reliable method is described to analyze different NK cell functions using a flow cytometry-based assay. NK cell functions can be evaluated not only for the whole NK cell population, but also for different NK cell subsets. This technique enables scientists to easily study NK cell functions in healthy donors or patients in order to reveal their impact on different malignancies and to further discover new therapeutic strategies.

Video Link

The video component of this article can be found at https://www.jove.com/video/54615/

Introduction

As part of the innate immune system natural killer (NK) cells contribute to the first line of defense against virus-infected or malignantly transformed cells. A system of inhibitory and activating receptors enables them to distinguish between healthy and transformed cells without prior antigen priming in contrast to T cells. Upon target cell encounter NK cells release the content of their cytotoxic granules (*e.g.,* perforin, granzymes) into the immune synapse to kill their target. Moreover, NK cells produce and secrete different kinds of cytokines (*e.g.,* interferon-γ: IFN-γ; tumor necrosis factor-α: TNF-α) and chemokines (*e.g.,* macrophage inflammatory protein-1β: MIP-1β) upon target cell interaction or cytokine stimulation[1].

Sufficient NK cell functions such as cytotoxicity, chemokine and cytokine production have an important impact on the fate of diverse diseases. Leukemia patients show increased relapse rates if they exhibit a defective NK cell profile at diagnosis consisting of reduced IFN-γ production and reduced expression of activating NK cell receptors[2]. An early recovery of NK cell numbers and function including cytokine production upon target cell interaction is associated with a reduced relapse and improved survival rate in patients receiving allogeneic stem cell transplantation[3]. Moreover, upon initiation of interferon therapy in hepatitis C virus-infected patients the degranulation capacity of peripheral NK cells is stronger in early responders than in non-responders[4]. NK cell numbers (>80/µl) on day 15 after autologous stem cell transplantation (autoSCT) in patients suffering from lymphoma or multiple myeloma are predictive for an improved progression free and overall survival[5]. In melanoma patients the expression of the T-cell immunoglobulin- and mucin-domain-containing molecule-3 (TIM-3), an immune-regulatory protein on NK cells, correlates with disease stage and prognosis[6].

Scientists have monitored NK cell functions throughout the last decades. The initial analysis of NK cell cytotoxicity against tumor cells without prior priming was addressed using a ^{51}Cr-release assay[7]. More recently, scientists developed a non-radioactive method to evaluate the cytotoxicity of expanded NK cells[8]. Cytokine and chemokine production has been frequently evaluated using enzyme-linked immunosorbent assay (ELISA) techniques[9,10]. During the last decades these methods have been complemented by flow cytometry-based assays. The use of protein transport inhibitors (*e.g.,* brefeldin A and monensin) and cell permeabilization methods in combination with conventional surface staining protocols have enabled scientists to study chemokine and cytokine production in different specific lymphocyte subsets (*e.g.*, T, B or NK cells)[11]. Moreover, different flow cytometry-based assays have been developed to monitor T and NK cell cytotoxicity. In 2004 Alter *et al.* described the surface expression of the lysosome-associated protein CD107a (Lamp1) on NK cells upon target cell encounter as a marker for the degranulation of cytotoxic granules[12]. Since a wide range of different fluorochromes and multi-channel flow cytometers are available in our days, it has become possible to simultaneously monitor diverse NK cell functions (cytotoxicity, cytokine and chemokine production) in different

NK cell subsets. This becomes especially important in situations where sample size is limited, *e.g.*, in biopsies or blood samples of patients suffering from leukopenia.

To test global NK cell functions, the different flow cytometry-based assays can be efficiently combined. Theorell *et al.* stimulated NK cells from healthy donors with the tumor cell line K562 and analyzed NK cell degranulation, inside-out signal and chemokine production via flow cytometry[13]. Recently NK cell subgroups, phenotypes and functions in tumor patients during autoSCT were analyzed using flow cytometry-based assays. It was demonstrated that NK cells were able to degranulate and produce cytokines/chemokines upon tumor cell recognition at very early time points after autoSCT[11].

Here a protocol is described to evaluate NK cell functions upon interaction with tumor cells including degranulation capacity, chemokine and cytokine production using a flow cytometry-based assay that makes it possible to monitor NK cell functions in different subsets simultaneously.

Protocol

This study was carried out in accordance with the recommendations of the local ethics committee of the University of Frankfurt.

1. Culturing of K562 Cells

1. Culture K562 cells in R10 media (RPMI1640 with glutamine medium, 1% penicillin/streptomycin, 10% fetal calf serum) at a density of 0.5-1 x 10^6 cells per ml in a cell culture flask at 37 °C and 5% CO_2.
2. Harvest K562 Cells 24 hr Before the Start of a New Experiment.
    1. Remove the cell culture flask containing the K562 cells from the incubator. Re-suspend the K562 cells within the culture media by gently pipetting up and down.
    2. Transfer the culture media containing the K562 cells into a 15 or 50 ml tube and pellet the cells at 400 x g for 8 min. Discard the supernatant and re-suspend the cells in 5 ml R10 media and mix well.
    3. Transfer 20 µl of the cell solution into a well of a 96-U-well plate. Add 20 µl trypan blue and mix well by pipetting up and down for at least 5 times. Pipette 10 µl of the solution into a cell counting chamber and count the cells.
    4. Pellet the cells at 400 x g for 8 min. Re-suspend the cells in R10 media and adjust the K562 cell concentration to 0.5-1 x 10^6 cells per ml.
    5. Incubate the K562 cells in a cell culture flask at 37 °C and 5% CO_2 until use.

2. Isolation of NK Cells

1. According to the approval and guidelines of the local ethics committee, obtain written informed consent of the healthy donor or patient before the collection of 6-10 ml of either EDTA or heparinized peripheral blood.
2. Store reagents for the NK cell isolation at 4 °C until the start of the experiment and then use them at room temperature (RT) under sterile conditions. Isolate NK cells from peripheral blood using magnetic microbeads and a specific magnet for cell isolation according to the manufacturer's instructions.
    1. Reconstitute one vial of the lyophilized NK cell negative isolation antibody cocktail by pipetting 7.5 ml of the provided buffer A (included within the NK cell isolation kit) into the vial. Mix gently by pipetting up and down 3-4 times.
    NOTE: Make sure, that the suspension is homogenous before each use.
    2. Prepare the final cocktail solution by mixing appropriate volumes of the reconstituted pellet from step 2.2.1 and buffer B (included within the NK cell isolation kit). To determine these volumes, define the volume of the whole blood sample in ml as volume = 1.0. Use 0.25 volumes of the reconstituted pellet (from step 2.2.1) and 0.25 volumes of buffer B to process 1 volume of whole blood.
    3. Transfer the mixture into an adequate tube containing the whole blood sample. Do not pipette up and down to avoid the loss of blood within the pipette. Instead, close the tube and move it carefully up and down until the suspension is homogenous. Do not vortex.
    4. Further homogenize the sample using a tube rotator for 5 min at RT.
    5. Under sterile conditions, remove the cap and place the tube into the magnetic separator for 15 min as recommended by the manufacturer. Make sure that the tube labels face towards the backside of the magnet to allow undisturbed visibility of the separation line. Do not move the magnet during separation, as this procedure would be disturbed.
    6. Carefully transfer the supernatant into a new 15 ml tube and fill up with complete media (CM; hematopoietic cell media, 1% penicillin/streptomycin, 5% human sera). Pellet the cells at 400 x g for 8 min.
        1. Optional: If the pellet is red, re-suspend the cells in 1ml of erythrocyte lysis buffer (*e.g.*, 1 ml of ammonium-chloride-potassium (ACK) lysing buffer: 155 mM NH_4Cl; 0.1 mM EDTA; 10 mM $KHCO_3$ in 1 L distilled water (DW)) and incubate for 8 min at RT. Alternatively, use an erythrocyte depletion kit.
        NOTE: Keep in mind, that the use of ACK buffer might negatively impact NK cell functions[14].
        2. Rapidly dilute the ACK buffer by adding at least 1 ml of CM to stop the lysis and centrifuge at 400 x g for 8 min to pellet the cells.
    7. Re-suspend the cell pellet in 1 ml CM and proceed with the counting. Transfer 20 µl of the NK cell solution onto a 96-U-well plate. Add 20 µl of methylene blue to each well and mix well by pipetting up and down for at least 5 times. Pipette 10 µl of the solution into a cell counting chamber and count cells. Alternatively, use 30 µl of undiluted cell suspension for counting with a commercially available cell counter.
    8. Adjust the cells to a concentration of at least 2 x 10^4 NK cells per 100 µl CM.
    9. Perform a surface antibody staining of the isolated NK cell population to check the purity using a flow cytometer.
        1. Prepare a master mix solution by mixing the volumes of the indicated antibodies as per **Table 1**.
        2. Re-suspend the cells in 88.5 µl washing buffer (WB; 0.5% bovine serum albumin (BSA), 0.1% NaN_3 in PBS) and add 11.5 µl of the master mix solution. Incubate the cells for 20 min at 4 °C in the dark.

Caution: NaN_3 is toxic and mutagen. Handle it accordingly to the corresponding Material Safety Data Sheet (MSDS).
3. Add 1 ml of WB and pellet the cells at 400 x g for 4 min.
4. Re-suspend the cells in 300 µl staining buffer plus DAPI. Acquire the cells using a flow cytometer.
NOTE: Proceed further with the experiment only if the NK cell purity is at least 80% of all alive lymphocytes.

3. Harvesting of the K562 Cells for NK Cell Stimulation

1. Remove the cell culture flask containing the K562 cells (from step 1.2) from the incubator. Re-suspend the K562 cells within the culture media by gently pipetting up and down.
2. Transfer the whole culture media into a 15 or 50 ml tube and pellet the cells at 400 x g for 8 min. Discard the supernatant and re-suspend the cells in 5 ml phosphate-buffered saline (PBS) and mix well.
3. Transfer 20 µl of the cell solution into a well of a 96-U-well plate. Add 20 µl trypan blue and mix well by pipetting up and down for at least 5 times. Pipette 10 µl of the solution into a cell counting chamber and count the cells. Alternatively use 30 µl of undiluted cell suspension to count with a commercially available cell counter.
4. Pellet the cells at 400 x g for 8 min. Re-suspend cells in CM at a concentration of at least 2 x 10^4 K562 cells per 100 µl media.
NOTE: Use the same K562 and NK cell concentrations in order to incubate both at an effector:target (E:T) ratio of 1:1 later on.

4. Stimulation of NK Cells with the Tumor Cell Line K562 and Cytokines

1. Gently mix the cells by pipetting them up and down for at least 5 times. Distribute 100 µl/well of the NK cell solution into the required wells of a 96-V-well plate.
    1. Use at least two wells for each donor, one with and one without a stimulus (*e.g.*, K562 tumor cells and cytokines). Whenever possible, perform the experiment at least in duplicate and add a positive control (*e.g.,* phorbol 12-myristate 13-acetate (PMA) and ionomycin; final concentration: 50 nM PMA, 1 µM ionomycin per well). Prepare updated flow cytometry compensations for the date of the experiment.
    NOTE: Titrate the antibodies prior use (see discussion).

2. Switch off the light and add the anti-CD107a antibody (2 µl, final concentration: 1:100) to each well with NK cells on the 96-V-well plate.
3. Gently mix the K562 cells by pipetting them up and down for at least 5 times.
    1. From the wells containing the NK cell/anti-CD107a antibody solution, identify the ones planned for stimulation with K562 cells. Add 100 µl/well of the K562 cell solution into these wells. Mix carefully by pipetting up and down for at least 5 times.
    2. Add interleukin-2 (IL-2; final concentration 100 U/ml) and interleukin-15 (IL-15; final concentration 10 ng/ml) to the wells containing K562 cells and the NK cell/anti-CD107a antibody solution.
        1. Optional: Test the impact of either K562 cells or cytokines alone on the distributed NK cells to decipher tumor-induced versus cytokine-induced NK cell functions.

    3. Identify the wells on the 96-V-well plate planned as negative controls without stimulus. Add 100 µl/well CM to these wells containing only the NK cell/anti-CD107a antibody solution. Mix carefully by pipetting up and down for at least 5 times.
        1. Optional: For a positive control, add 100 µl CM containing PMA and ionomycin (final concentration: 50 nM PMA, 1 µM ionomycin per well) to a well with the NK cell/anti-CD107a antibody solution.

4. Incubate the Cells for 3 hr in the Dark at 37 °C and 5% CO_2.
5. Prepare a protein transport inhibitor solution (*e.g.,* brefeldin A and/or monensin) in CM. After 1 hr of incubation, add the solution to each well of the 96-V-well plate with the light switched off (final concentration: 0.5-1µM brefeldin A and 2-3 µM monensin). Mix carefully by pipetting up and down for at least 5 times. Continue the incubation for the remaining 2 hr.

5. Surface and Intracellular Staining

1. After the 3 hr incubation period, mix the cells within the wells of the 96-V-well plate carefully by pipetting up and down for at least 5 times and transfer them into flow cytometry tubes. Add 1 ml/tube of WB. Pellet cells at 400 x g for 4 min.
    1. Optional: Before centrifugation rinse the wells with 100 µl/well PBS, in order to optimize the cell recovery, by pipetting up and down for at least 5 times before transferring the cells into the respective FACS tube.

2. Discard the Supernatant and Continue with the Surface Staining.
3. Include an amine-reactive fluorescent dye that is non-permeant to live cells with an emission maximum of 423 nm as a fixable dead cell marker (DCM). Perform the staining before (see below) or in parallel with the antibody surface staining depending on the type of the DCM used.
    1. Re-suspend cells in 99 µl/tube PBS and add 1 µl/tube of the fixable DCM (final concentration: 1:100). Mix cells well using a vortex and incubate them for 15 min at RT in the dark.
    2. Prepare a master mix solution by mixing together the "surface" antibodies listed in **Table 2** (see staining step column highlighted in blue). Use the indicated volumes/tube and multiply them with the number of flow cytometry tubes to be stained.
    3. After incubation take the flow cytometry tubes with the cells and add 1 ml/tube of WB. Pellet the cells at 400 x g for 4 min.
    4. Discard the supernatant, re-suspend the cells in 84 µl/tube of WB and add 16 µl/tube of the master mix solution. Mix cells well using a vortex. Incubate cells for 20 min at 4 °C in the dark.
    5. After 20 min add 1 ml/tube WB and pellet cells at 400 x g for 4 min.

4. Discard the supernatant and re-suspend the cells in 100 µl/tube of a cold fixation solution (*e.g.,* 2% (final) paraformaldehyde or formaldehyde). Mix cells well using a vortex. Incubate cells for 10 min at 4 °C in the dark.
    1. Stain the cells for intracellular cytokines/chemokines after this step or store them overnight at 4 °C in WB.
    NOTE: After overnight incubation, a lower cell recovery may occur.
    Caution: Paraformaldehyde and formaldehyde are potential carcinogens. Handle them accordingly to the respective Material Safety Data Sheet (MSDS).

5. Wash cells in 1 ml/tube WB at 400 x g for 4 min and proceed with the permeabilization step.
6. Prepare a permeabilization buffer (PB) (*e.g.,* 0.2% saponin, 1% BSA solution in PBS).
7. Wash the cells twice in 1 ml/tube of PB at 400 x g for 4 min. Continue with the intracellular staining.
Caution: Saponin is potentially irritating. Handle it accordingly to the corresponding Material Safety Data Sheet (MSDS).
8. Prepare a master mix solution using the indicated "intracellular" antibody volumes in **Table 2** (highlighted in green). Multiply these volumes with the number of tubes to be stained.
    1. Re-suspend the cells in 90 µl/tube PB and apply 10 µl/tube of the antibody master mix solution. Mix well using a vortex and incubate the cells for 30 min at 4 °C in the dark.

9. Wash Cells Twice in 1 ml/tube of PB at 400 x g for 4 min.
10. Discard the supernatant and re-suspend the cells in 400 µl/tube PB. Mix cells well using a vortex. Place cells on ice in the dark until measurement.

6. Flow Cytometry Compensation and Acquisition

1. Select the channels for the different fluorochromes (see **Table 2**) within the flow cytometry acquisition software in the parameter setup folder. Additionally, choose the channels for FSC-A and -H as well as for SSC-A and -H.
2. Load a compensation matrix created for the chosen parameters into the acquisition file.
    1. Create a compensation matrix using isolated NK cells from step 2.2.8 by performing a single color stain for each used fluorochrome (see **Table 2**). Use the surface staining protocol described in steps 5.1-5.4. Include an unstained control (without any antibodies) as well as isotype controls and/or fluorescence minus one controls (FMO).
    NOTE: As an alternative to cell-based compensation, use IgG-binding beads as compensation controls.
    2. Place each tube for single stain samples and the sample without antibodies into the sample injection port (SIP). Click on the "record" button in the flow cytometry acquisition software and acquire a cell number of at least 5 x 10^3 NK cells per sample.
    3. Use the compensation calculation application of the cytometry acquisition software to create a compensation matrix.

3. Create Dot Plots for Identifying the NK Cell Population.
    1. Identify single cells using FSC-A/FSC-H and SSC-A/SSC-H dot plots. Click on the "polygon gate" button. Draw a gate around cells, which have a linear distribution pattern in both dot plots. Double-click on the gates to open a new dot plot.
    2. Choose a FSC-A/SSC-A dot plot to identify the lymphocyte/NK cell population (see **Figure 1**). Draw a polygon gate around it and double-click on the gate.

4. Place each sample tube of the NK cell stimulation assay into the SIP. Click on the "record" button and acquire a cell number of at least 5 x 10^3 NK cells per sample.

7. Analysis and Statistics

1. Open the flow cytometry analysis software program. Click on the loading sample button to open the unstimulated control sample.
2. Create a Gating System for the Different NK Cell Subpopulations (see **Figure 1**).
    1. Click on the dot plot button to create a FSC-A/SSC-A plot. Click on the rectangle gate button and draw a rectangle gate over all events with a FSC-A value >5 x 10^4 to exclude debris. Open the gate by double-clicking on the gate.
    2. Choose again the FSC-A/SSC-A parameters within the new dot plot and click on the polygon gate button. Draw a polygon gate around the lymphocyte population (see **Figure 1**). Open the gate.
    3. Select the SSC-A/SSC-H parameter for the new dot plot and draw a polygon gate around all single cells. Single cells demonstrate a linear distribution across the FSC-A/FSC-H and SSC-A/SSC-H parameters (see **Figure 1**). Open the gate by double-clicking on it.
    4. Repeat step 7.2.3 using the FSC-A/FSC-H parameters this time.
    5. Use the parameter CD45 and Dump channel (CD3; CD14; CD19; DCM) to exclude dead cells. Draw a rectangle gate around all CD45 positive and Dump channel negative cells. Open the gate by double-clicking on the gate.
    6. Identify the whole NK cell population by using the CD56 and Dump channel parameters. Create a rectangle gate around the CD56 positive and Dump channel negative cells. Open the gate by double-clicking on it.
    7. Draw a rectangle gate around all the three NK cell subsets within a CD56/CD16 dot plot. Identify the different subsets as $CD56^{++}CD16^{-}$, $CD56^{++}CD16^{+}$ and $CD56^{+}CD16^{++}$ NK cell subsets.

3. Analyze NK Cell Functions for Each Individual NK Cell Subset.
    1. Click on one of the three NK cell subset gates to open it. Create three different dot plots for CD56/CD107a, CD56/IFN-γ and CD56/MIP-1β.
    2. Draw a rectangle gate for CD56 positive cells, which are positive as well for CD107a, IFN-γ or MIP-1β respectively (see **Figure 1**).
    3. Repeat step 7.3.1-7.3.2 for each remaining NK cell subset.

4. Repeat step 7.2 and 7.3 for all stimulated samples. Save all statistical values of each individual sample by clicking on the statistic-exporting button within the analysis software.

5. Open the files containing the statistical values of the individual samples to analyze NK cell functions. Select the values for individual NK cell subsets (*e.g.*, $CD56^{++}CD16^{-}$ NK cells) by copying them into another file.
    1. Subtract the percentage of CD107a positive NK cells, which have not been treated with a stimulus, from the percentage of CD107a positive NK cells, which have been treated with a stimulus.
    NOTE: Use the result to demonstrate the degranulation capacity of this particular NK cell subset in response to the stimulus.
    2. Repeat step 7.5.1 for IFN-γ and MIP-1ß-positive NK cells to demonstrate the cytokine and chemokine production in response to the stimulus.
    3. Repeat step 7.5.1-7.5.2 for each remaining NK cell subset to evaluate their degranulation capacity and cytokine/chemokine production upon stimulation.

Representative Results

The gating strategy for analyzing the degranulation, cytokine and chemokine production of the whole NK cell population and three different NK cell subsets are illustrated in **Figure 1**.

Representative results of one healthy donor are illustrated in **Figure 2**. NK cells without any stimulus produced neither IFN-γ nor MIP-1β and did not express CD107a on their surface (**Figure 2A**). In contrast, NK cells stimulated with tumor cells and cytokines produced significant amounts of intracellular IFN-γ and MIP-1β. Moreover, over 20% of them degranulated upon tumor cell interaction indicated by expressing CD107a on their surface (**Figure 2C**). PMA and Ionomycin stimulation were used as a control (**Figure 2B**).

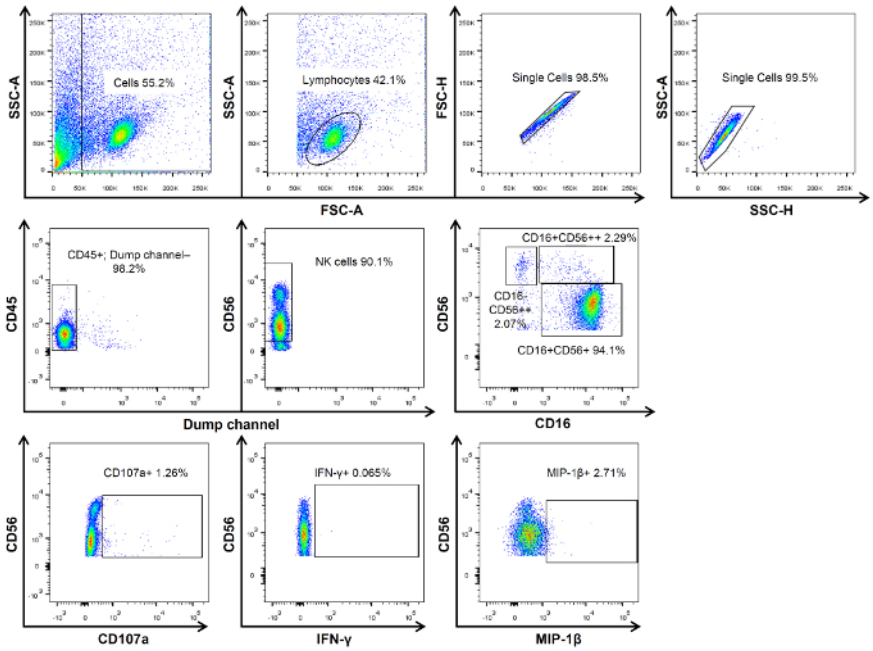

Figure 1: Gating strategy. Subsequent gates are plotted. First, debris are excluded in a FSC-A/SSC-A plot. Then lymphocytes are identified and doublets are excluded using two plots with SSC-A/SSC-H and FSC-A/FSC-H. Thereafter, a gate including all $CD45^{+}$ cells and excluding all dead, $CD3^{+}$, $CD14^{+}$ and $CD19^{+}$ cells is set by plotting CD45 / Dump channel. Next, NK cells are identified by gating on $CD56^{+}$ cells. A plot with CD56/CD16 can be used to identify the main NK cell subsets. Finally, within the whole NK cell population, functional markers are identified by plotting CD56 versus CD107a, IFN-γ and MIP-1β, respectively. This can be done as well for all different kinds of NK cell subsets. Please click here to view a larger version of this figure.

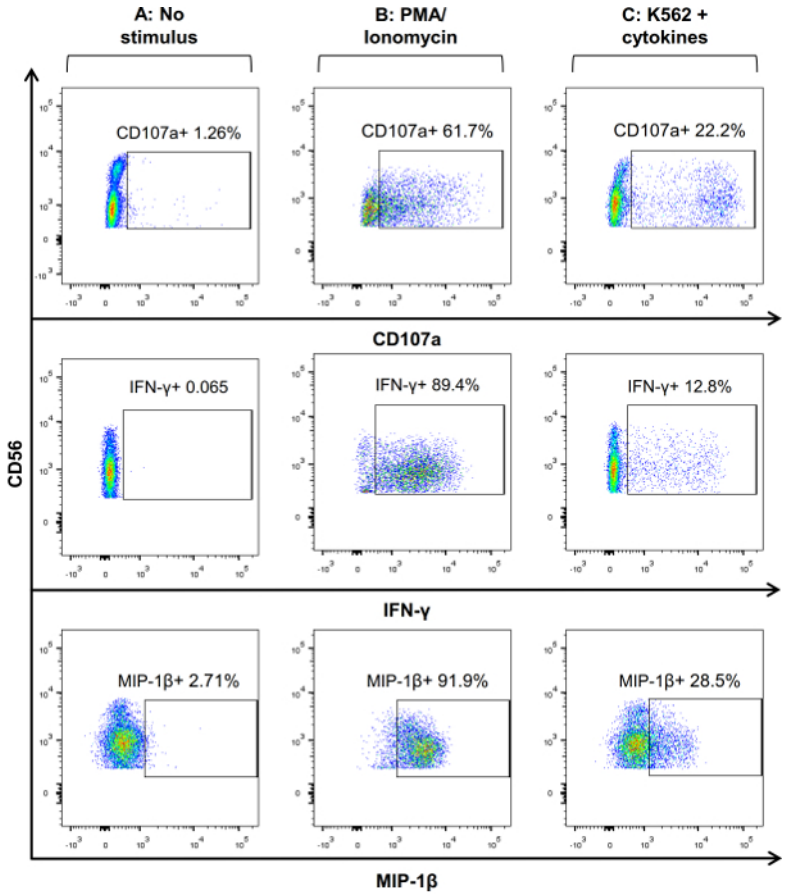

Figure 2: Representative results from one healthy NK cell donor. Using the gating strategy described in **Figure 1**, NK cell functional markers like CD107a (for degranulation), IFN-γ and MIP-1β can be identified. The left column (**A**) shows the results using NK cells in the absence of a stimulus. The middle column (**B**) demonstrates the results in the presence of the positive control PMA/ionomycin. The right column (**C**) presents results for NK cells stimulated with the tumor cell line K562 and in the presence of the cytokines IL- 2 (100 U/ml) and IL-15 (10 ng/ml). Please click here to view a larger version of this figure.

Filters	Fluorochrome	Marker	Staining step	Dilution	Volume/tube used for mastermix solution
530/30	FITC	CD56	Surface	1:25	4 µl
575/25	PE	CD16	Surface	1:200	0.5 µl
695/40	PerCP	CD14	Surface	1:25	4 µl
780/60	PE-Cy7	CD19	Surface	1:100	1 µl
670/30	APC	CD3	Surface	1:100	1 µl
712/21					
780/60					
450/50	DAPI	dead cells	Before measurment	1:6000	/
525/50	BV510	CD45	Surface	1:100	1 µl
605/40					

Table 1: Antibodies for purity check.

Laser (nm)	Dichroic Mirror	Filters	Fluorochrome	Marker	Staining step	Dilution	Volume/tube used for mastermix solution
488	502	530/30	FITC	CD56	surface	1:25	4 µl
488	550	575/25	PE	CD107a	in well	1:100	/
488	655	695/40	PerCP	CD16	surface	1:20	5 µl
488	735	780/60					
635	blank	670/30	Alexa Fluor 647	IFN-gamma	intracellular	1:20	5 µl
635	685	712/21					
635	735	780/60	APC-H7	MIP1-beta	intracellular	1:20	5 µl
405	blank	450/50	fixable dead cell marker	dead cells	fixable dead cell staining	1:100	/
405	blank	450/50	V450	CD3/CD14/CD19	surface	1:100/1:25/1:100	1 µl/4 µl/1 µl
405	505	525/50	BV510	CD45	surface	1:100	1 µl
405	570	605/40					

Table 2: Antibodies for extra- and intracellular staining.

Discussion

The described method is an easy, fast and reliable approach to study NK cell functions from whole blood samples of healthy donors or patients. This method offers the great advantage to directly purify NK cells from whole blood, avoiding the time-consuming density gradient centrifugation, which is mandatory for many other purification methods[15]. Moreover, it requires a smaller sample size compared to "classical" NK cell isolation/ enrichment methods, which makes it a suitable alternative for samples of pediatric and/or immune-deficient patients. This protocol can be used to obtain basal values of NK cell functions *ex-vivo*, but it also allows the further NK culture and expansion, being in this way complementary with other methods previously described[8]. Nevertheless some critical steps have to be taken into account. Since the assay is based on extra- and intracellular antibody staining, it is crucial to determine the optimal working concentration for each antibody first. Especially for the intracellular staining, a careful evaluation of the used antibodies is highly recommended. A good approach is to test a dilution series of the used antibody (*e.g.,* 1:100 to 1:12.5) in a cell suspension stimulated with or without PMA/ionomycin. Subsequently the ratio of positive events between stimulated and un-stimulated samples are calculated and plotted. The antibody dilution with the highest positive fold change ratio (best signal-to-background ratio) should be used for further experiments[16]. Moreover, the use of controls like isotype- and FMO controls can prove to be fundamental especially for intracellular staining in order to gate correctly on the positive cell populations.

However, there are some important limitations of the described method. CD107a expression is a degranulation marker and therefore only indirectly indicates NK cell cytotoxicity. NK cell cytotoxicity and degranulation capacity can be different. Up-regulation of the autophagy pathway in tumor cells results in the degradation of secreted granzyme b and reduces cell death upon NK cell interaction[17]. Therefore an additional DCM (*e.g.,* cleaved caspase 3) might be added to the staining panel in order to monitor cell death events within the target cells[18]. Additionally, NK cell cytotoxicity is influenced by the amount of cytotoxic proteins within their granules (*e.g.*, perforin, granzyme b), which can be quantified by an intracellular staining[19,20].

Furthermore, there are different possibilities to modify the protocol. Instead of isolated NK cells, peripheral blood mononuclear cells (PBMCs) can be used. Though one has to be aware that the absolute NK cell number within the PBMC population differs between various donors and even between samples derived from the same donor at different time points. NK cell degranulation is highly dependent on the E:T ratio. In order to compare NK cell functions between different donors or at various time points, the absolute NK cell number within the PBMC population should be used to establish a constant E:T ratio throughout all experiments. If NK cell functions are monitored at different time points within the same donor, PBMCs can be frozen and the analysis can be done at once for all different time points in order to reduce inter-experimental variations[11].

Since staining panels with up to 18 colors are possible, analysis of NK cell functions can be extended to a much greater detail. Using additional surface markers including CD57, NKG2A and killer cell immunoglobulin-like receptors (KIRs), further NK cell subsets can be identified. Educated NK cells expressing at least one self-KIR degranulate stronger upon interaction with K562 cells than uneducated NK cells. In contrast, more differentiated $CD57^{+}CD56^{+}$ NK cells produce less IFN-γ upon IL-12 and IL-15 stimulation compared to less differentiated $CD57^{-}CD56^{+}$ NK cells[21]. In addition, other markers of the NK cell function may be addressed. LFA-1 is an important molecule for NK cell adhesion and is expressed in its open conformation upon NK cell activation. This so-called "inside-out signal" is an early NK cell activation marker[22].

Finally, the stimuli for testing NK cell functions can be modified as well. In our experience freshly isolated NK cells only degranulate poorly upon K562 cell interaction if no cytokines are added. Using freshly isolated PBMCs without further cytokine addition circumvent this issue, because of the influence of bystander cells. Moreover, cytokine production, especially IFN-γ and TNF-α, can be initiated upon IL-12 and IL-18 stimulation[23]. IFN-γ production within the NK cell subsets may differ depending on the used stimulus (cytokine- versus target-induced stimulation)[24]. Additionally, instead of using K562 cells as target cells, primary tumor cells derived from tumor patients could be used in order to test NK cell functions against autologous tumor cells before or during immune-modulating therapies (*e.g.*, monoclonal antibody treatment or immuno-modulatory drugs (IMiDs)).

Within the past few years, the field of immunotherapy has been evolving rapidly with the clinical approval of immune checkpoint inhibitors (*e.g.,* ipilumumab, nivolumumab, pembrolizumab)[25] and successful trials using genetically modified chimeric antigen receptor (CAR)-expressing T cells as well as CAR-NK cells (reviewed in reference[26]), for the treatment of hematological tumor patients[27]. Therefore NK cell-based immunotherapy is getting increasingly more into focus. Anti-CD20 antibodies have been a great success in the treatment of malignant lymphomas within the last two decades[28]. The vast majority of NK cells express the low affinity Fc receptor CD16 enabling the cells to recognize and kill antibody-labeled target cells (antibody-dependent cellular cytotoxicity — ADCC). The antibody's ability to trigger NK cell functions can be tested *in vitro* using the described protocol[29]. Terszowski *et al.* analyzed NK cells' ADCC against a B lymphoblastoid cell line using different clinically approved anti-CD20 antibodies. While ADCC induced upon rituximab treatment (first clinically approved anti-CD20 antibody[30]) was negatively influenced by KIR/HLA interaction, this effect was not observed when using obinutuzumab (a novel glycoengineered type II CD20 monoclonal antibody)[31,32].

Moreover, NK cell functions are influenced by different novel anti-tumor drugs like the IMiD lenalidomide[33]and different tyrosine-kinase inhibitors (TKI)[34]. Currently NK cell specific checkpoint inhibitors are tested in clinical trials. Examples are the use of anti-KIR[35,36] or anti-NKG2A antibodies (NCT02331875) as well as activating agonists like an anti-CD137 antibody (NCT01775631; NCT02110082).

Importantly, adoptive NK cell transfer has been performed in a variety of different malignant diseases with promising clinical effects[37]. With the aim to select the best donor, NK cell functions against the patient's tumor might be tested *in vitro* using blood samples from potential NK cell donors.

In summary, the described protocol is designed to analyze diverse NK cell functions from healthy donors or patients. Those functions can be monitored in diverse NK cell subsets upon various stimuli at selected time points.

Disclosures

The authors have nothing to disclose.

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