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Leukocyte Reduction Filters Are Reliable and Economic Source for Natural Killer Cell Preparation

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ABSTRACT

Background: An issue that hinders researchers' access to Natural Killer (NK) cells is their low proportion in peripheral blood leukocytes. This issue is currently addressed by methods involving a series of differentiation and expansions that are time-consuming and expensive.

Objective: We have investigated whether the used leukocyte reduction filters, a by-product in the blood transfusion practice that currently is considered waste, can be utilized as a source of the NK cells.

Methods: Following the blood donation of 46 donors based on the Iranian Blood Transfusion Organization's protocols, a sample of peripheral blood of each donor and the leukocyte reduction filter used in their donation procedure have been obtained. The entrapped cells were flushed back from the leukocyte reduction filters. Both groups of samples were analyzed using an automatic hematological analyzer. NK cell isolation was done by the MACS negative selection method. The samples have been comparatively analyzed utilizing flow cytometry data of NK cells' subpopulation compositions, viability, degranulation patterns, and cytotoxic capacity against the K562 cell line.

Results: Every major leukocyte population was abundant in the samples extracted from the used leukocyte reduction filters. The NK cells extracted from leukocyte reduction filters did not show any statistically meaningful differences ($P<0.5$) from peripheral blood samples in terms of subpopulation composition, viability, degranulation potency, and cytotoxic capacity.

Conclusion: Used leukocyte reduction filters can be considered an economic, easy to obtain, and robust source of abundant research-grade NK cells.

Keywords: Cell isolation, Leukocyte reduction procedures, Natural killer cells, Peripheral blood mononuclear cells

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INTRODUCTION

NK cells play a pivotal role in many immune system functions in both states of health and disease. As a result, the demand for NK cells is rapidly increasing among researchers. Therefore, it is a valid concern to search for means to provide for this ever-increasing demand for NK cells. The researcher's access to NK cells is hampered by the fact that NK cells make up a minor proportion of a healthy person's peripheral blood mononuclear cells (PBMCs). This, combined with the fact that ethical issues limit the amount of blood that may be donated for research purposes, has pushed researchers to seek solutions to the low absolute count of accessible NK cells.

The most prevailing approach to overcome this issue is to perform ex vivo expansion using arrays of cytokines and genetically-modified feeder cell layers [1, 2]; This procedure is relatively expensive and time-consuming, and the expanded NK cells show signs of exhaustion and senescence. Another approach would be to establish or use established NK-based cell lines [3]; these cell lines may solve the problem of NK cell numbers in hand but raise other complications. The third approach is to generate NK cells from cord blood's homeopathic stem cells (HPSC) [4]; not only is cord blood not easily accessible to researchers, but the process of differentiating HPSC from the NK cells is also expensive and time-consuming. The proposed approaches are not limited to the aforementioned list, but they all share two characteristics: being expensive (requiring a considerable amount of reagents) and time-consuming.

To find a reliable source of PBMCs, a shift in perspective from clinical waste to a source of research-grade blood cell products has been proposed, but not yet widely incorporated, for the used leukocyte reduction filters. This idea is based on the fact that pre-storage leukocyte reduction is now a mandatory procedure in transfusion medicine [5]; the current generation of leukocyte reduction filters entrap roughly %99.9 of processed blood's leukocytes. Given the large volume of the processed blood unit (450 ml), each used leukocyte reduction filter contains a substantially high number of entrapped peripheral blood cells [6, 7].

Although some studies have reported the presence and successful recovery of NK cells from the entrapped cells in the leukocyte reduction filters, to our knowledge, none has comprehensively investigated the above-mentioned cells in a fashion that validates their suitability as a research-grade source of the NK cells. There is a multitude of criteria that must be assessed. In this research, we have tried to answer a question: can used leukocyte reduction filters be established as a source of research-grade NK cells? Furthermore, how do the NK cells obtained from this source compare against those obtained directly from the peripheral blood?

MATERIALS AND METHODS

Donors Recruitment and Sample Collection

This study was approved by the National Committee for Ethics in Biomedical Research of Iran (Ethic code IR.MODARES.REC.1398.027) and the Iranian Blood Transfusion Organization's (IBTO) Ethics Committee (Ethic code IR.TMI.REC.1397.008). IBTO's innovation center's staff carried out the recruitment of 46 healthy young male donors following the IBTO's internal regulations and code of conduct. Each donor has given written consent before the donation. The procedure of blood donation and subsequent processing and filtration was performed based on IBTO's standard operating procedure (SOP) without any modification or involvement of the researchers. Two samples have been delivered to us per every donation: 12 ml of pre-filtration peripheral blood, which served as the controls in our study design, and a used Macopharma LEUCOFLEX LST1 leukocyte reduction filter (Macopharma, France).

Filter Elution and WBC Extraction

Elution buffer composed of PBS containing 5 mM EDTA–Na2 and sucrose %2.5 [w/v] has been prepared based on Meyer et al. formulation [8] and subsequently stored at 4 °C. Two sterile syringes were attached to the filter's cords. The first syringe, attached at the counter-side of blood flow during filtration, was filled with 4 °C elution buffer, and the second syringe attached to the opposite chords of the filter was empty. By gently applying positive pressure from the elution buffer containing the syringe and negative pressure from the empty syringe at a constant rate, filter contents were drawn into the empty syringe. (Figure 1) Then, we injected the extracted suspension into a standard blood bag. (from Macopharma, containing 63ml CPDA-1 anticoagulant). We performed this process three times per filter with a total amount of 150 ml elution buffer.

Pre-processing and Density Gradient Isolation

For every sample, the blood bag's suspension transferred to three 50 ml conical centrifuge tubes, and the centrifugation was carried out at 800 ×g, 4 °C for 10 min. After the centrifugation, the tube's contents were fractionated into three layers. The middle white layer (buffy coat), which included PBMCs, was carefully extracted using a Pasteur pipette and transferred to another tube. PBMCs (including lymphocytes) were further isolated using Lymphodex Lymphocyte Separating Medium (Inno-train, Germany) according to the manufacturer's instructions.

Differential Cell Counting

After each PBMC extraction step, 1.5 ml samples from both peripheral blood and the suspension extracted from leukocyte reduction filters were obtained to be differentially counted using Sysmex automated hematology analyzer model 2000i.

Flow Cytometry Analysis of NK Cells' Subpopulations

After the density gradient isolation of PBMCs, a 100 µL sample of both the filter-extracted and peripheral blood suspension was collected. Using FITC-anti-CD3 antibody (Cat. No. 317305, BioLegend, USA), APC-anti-CD16 antibody (Cat. No. 302011, BioLegend, USA), and PE-anti-CD56 antibody (Cat. No. 362507, BioLegend, USA), the samples were stained and incubated in the dark for 30 min. Staining and sample preparation were performed based on the manufacturer's protocol. The samples were read using a BD FACSCalibur cell analyzer (BD Biosciences). The obtained results were analyzed using Flowjo v10 software (TreeStar Inc).

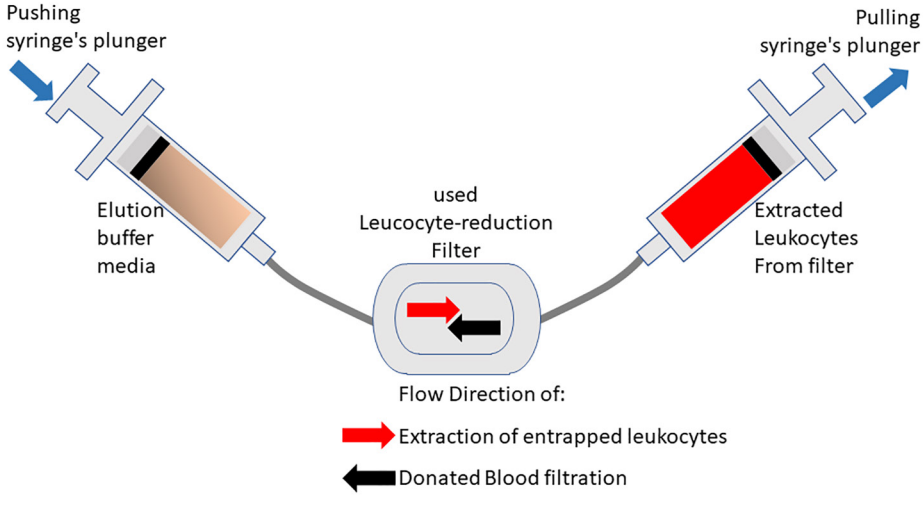

Figure 1. Schematic illustrations of extracting the WBCs entrapped in the leukocyte-reduction filters. Blue arrows are the direction of the applied forces.

NK Cells Isolation Using Negative Selection MACS

Following the manufacturer's instructions, the NK cells were isolated using negative selection MACS (MojoSort Human NK Cell Isolation Kit, Cat. No. 480053, BioLegend, USA). The isolated NK cells from both sources were subsequently transferred to a 15 ml conical tube containing RPMI 1640 culture medium and were incubated overnight. The success and purity of the NK cells isolation were confirmed using the flow cytometry against CD16 and CD56 cellular markers.

The K562 cell line (human chronic myelogenous leukemia) served as targets for the NK cell cytotoxicity effects. The K562 cell line was obtained from the Immunology Department of Tarbiat Modares University (courtesy of Dr. H. Sarraf, originating from the Iranian Biological Resource Center, cell no. IBRC C10081). Defreezing and cell culturing have been carried out according to standard protocols using RPMI 1640 %10 FBS medium. The cultured cells have been constantly monitored, and the passaging and sub-culturing procedure were performed based on standard guidelines and protocols.

Co-culture of the NK Cells with the K562 Cells

One day before each co-culture with the NK cell, the K562 culture media has been changed, and a volume of cells suspension equivalent to 10K cells has been transferred to 24 wells co-culture plate and was stained using CFSE dye (CFSE Cell Division Tracker Kit, Cat. No. 423801, BioLegend, USA). The co-culture procedure was then carried out by transferring a volume equivalent to 40K NK cells from both filters extracted and the control isolated NK cells suspension into the co-culture plate. Therefore, we set up a target-effector ratio of 1:4. The plate was then transferred to an incubation chamber. After 4 hrs., the co-culture was terminated, and a sample of the wells' content was obtained for the flow cytometry analysis.

Flow Cytometry Analysis of NK Cells and the K562 Cells Co-culture

Before the co-culture, the K562 cells were stained using CFSE (BioLegend CFSE Cell Division Tracker Kit, USA) according to the manufacturer's instruction; subsequently, the co-culture was initialized as stated above. For the wells intended for degranulation analysis, the APC ANTI-CD107a antibody (Cat. No. 328619, BioLegend, USA) was immediately added. After 4 hrs., the wells that contained CD107a antibody were immediately read using a BD FACSCalibur cell analyzer (BD Biosciences). Other wells were stained using PE Annexin V Apoptosis Detection Kit with 7-AAD (Cat. No. 640934, BioLegend, USA) according to the manufacturer's instructions and were read using BD FACSCalibur cell analyzer (BD Biosciences). The obtained results were analyzed using Flowjo v10 software.

Statistics

To analyze the differential cell counts and flow cytometry of CD3, CD16, and CD56 data, the Shapiro-Wilk test was used to confirm normal distribution. If $P>0.05$, we then proceeded to perform paired T-test. For the flow cytometry analysis data of CD107a annexin V, and 7-AAD data where N=3, the Wilcoxon signed-rank test (non-parametric alternative of paired t-test) was performed. The P-value threshold for all the tests was set to 0.05. All the analysis was done using the GraphPad Prism software v9 (GraphPad Software Inc).

RESULTS

Differential Cell Counting

Using the differential cell count function of the Sysmex automated Hematology analyzer device, we analyzed WBCs extracted from each of our 46 donor's peripheral blood (the control group) and used leukocyte reduction filters. This step was done to examine the recovery process efficiency and populational composition of

the obtained cell suspensions. The measured concentrations of WBC and neutrophils, lymphocytes, monocytes, eosinophils, and basophils concentration in both groups of samples are summarized in the first two columns of Table 1. The detailed dataset is available in supplementary file, sheet 1.

Recovery rates data for total WBC and each population is summarized in the third column of Table 1 and the second sheet supplementary file. Our target subset is the lymphocytes since they encompass the NK cells. Note that the average lymphocyte recovery rate (29.12 12.18) is higher than the recovery rates of other WBC populations and the total WBCs in general. Also, as shown in Figure 2 and Table 1, the average composition of WBCs in the peripheral blood and leukocyte reduction filters have some statistically significant differences.

Table 1. Differential cell counts results

	Cellular concentrations (in 10^6 cell/ mL)		Recovery Rate (%)	Cellular Composition (%)		Obtainable Cells
	PB (Mean±SD)	LF (Mean±SD)	(Mean±SD)	PB (Mean±SD)	LF (Mean±SD)	(Mean±SD)
WBC	7.01±2.37	3.75±1.67	18.38±7.33	-	-	5.62E+08±2.50E+08
NEUT	3.98±1.77	1.57±1.01	13.32±7.02	56.04±8.77	41.19±16.84	2.36E+08±1.51E+08
LYMPH	2.21±0.70	1.76±0.79	28.13±12.19	32.23±7.60	48.05±14.97	2.64E+08±1.18E+08
Mono	0.55±0.19	0.28±0.16	18.67±11.89	7.96±2.17	7.31±3.00	4.15E+07±2.45E+07
EO	0.23±0.17	0.19±0.14	29.24±20.22	3.29±1.84	4.99±3.40	2.80E+07±2.06E+07
BASO	0.04±0.03	0.01±0.01	15.99±12.86	0.55±0.48	0.42±0.49	2.12E+06±1.64E+06

The first two columns are cellular concentrations (in 10^6 cells/ mL) of Leukocytes in the samples obtained from peripheral blood (PB) and leukocyte reduction filters (LF). The third column is the Recovery rates of Leukocytes obtained from leukocyte reduction filters with reference to what is expected from 450 ml of the donor's peripheral blood; the numbers have been calculated from differential cell counts of each donor's peripheral blood sample. Since the cell contents of 450 ml peripheral blood was collected into 150 ml elution buffer, to adjust for concentration changes, the numbers have been divided by three. Next are the cellular composition within LF and PB samples, based on differential cell counts. The last column is the estimated counts of leukocytes obtainable from one leukocyte reduction filter elution. The full dataset is presented in the supplementary file 1. (WBC=White blood cells, NEUT=Neutrophils, LYMPH=Lymphocytes, MONO=Monocytes, EO=Eosinophils, BASO=Basophils)

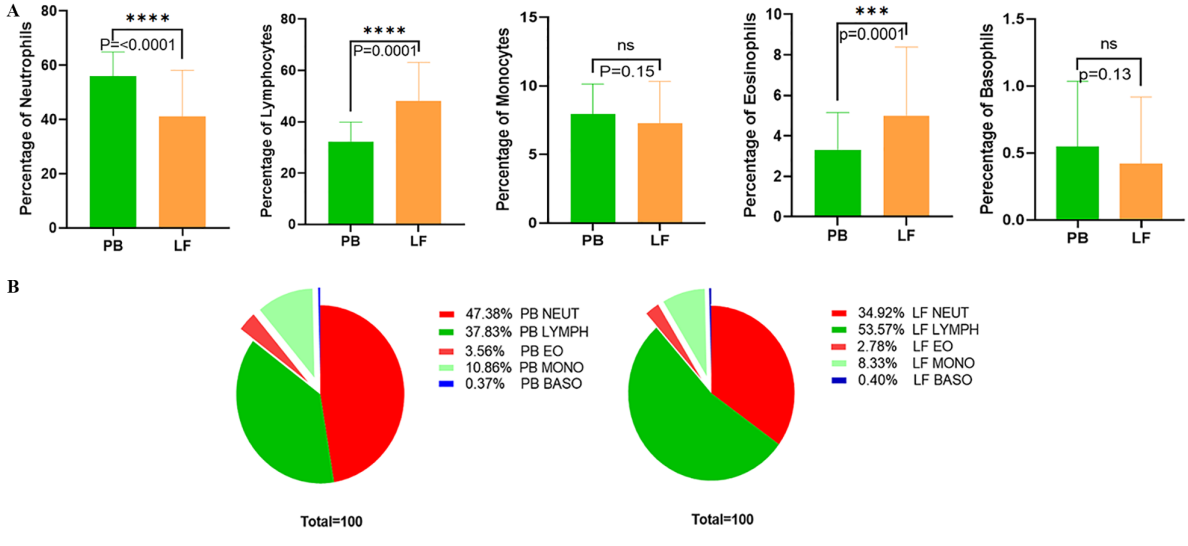

Figure 2. A) Statistical comparison of each leukocyte major population in samples obtained from peripheral blood (PB) and used leukocyte-reduction filters (LF). The statistical significance was calculated by paired T-test. (N=46, ****P<0.00005, ***P<0.0005, ns=not significant). B) Distribution of each Leukocyte major population in samples obtained from peripheral blood (PB) and used leukocyte-reduction filters (LF)

The most notable is the over-representation of lymphocytes percentage among WBCs (from 32.22±7.60 in the peripheral blood to 48.05±14.97 in leukocyte reduction filters, with P<000.1) Neutrophils are also depleted (P<0.0001), but eosinophils' percentage among the WBCs shows a slight increase (P=0.0001). The trend for monocytes and basophils was not statistically significant. The high natural variability of human WBC compositions is observed in the peripheral blood cell count data and reflected in the leukocyte reduction filter data.

In the last column of Table 1, we estimated the absolute cell numbers that could be extracted from a leukocyte reduction filter based on the cell count data. Note that the average estimated absolute count for WBC is 5.62E+08±2.50E+08 and for lymphocytes is 2.64E+08±1.18E+08.

NK Cells Subpopulation Analysis (Flow Cytometry of CD3, CD16, and CD56)

We utilized the flow cytometry analysis of CD3, CD16, and CD56 surface markers to examine the differences in the NK cell subpopulation compositions between peripheral blood and filter-extracted suspension in 13 of the donors. The analysis results are shown in Table 2 and Figure 3. The paired analysis revealed no statistically significant differences in the NK cells' three major populations, $CD56^{+}CD16^{-}$, $CD56^{+}CD16^{+}$, and $CD56^{-}CD16^{-}$, between the samples extracted from leukocyte reduction filters and peripheral blood. (N=13, P respectively: 0.100, 0.228, and 0.080, all distributions were normally based on the Shapiro-Wilk test.). The results confirm that extracting the NK

Table 2. Flow cytometry analysis of the NK cells' subpopulations

Sample	%LYMPH	$\%CD3^{-}$	$\%CD56^{+}CD16^{-}$	$\%CD56^{+}CD16^{+}$	$\%CD56^{-}CD16^{+}$	%NK cells in $CD3^{-}$	%NK cells in lympH
Donor 1 PB	90.6	28.5	7.7	31.3	37.4	21.8	19.7
Donor 2 PB	89.1	36.8	7.7	40.4	34.2	30.3	27.0
Donor 3 PB	86.2	40.1	3.3	36.08	43.2	33.1	28.5
Donor 4 PB	60.7	35.5	4.3	49	33.7	30.9	18.7
Donor 5 PB	86.1	40.8	0.9	43.9	39.4	34.4	29.6
Donor 6 PB	85.2	39.1	1.37	2.6	75.7	31.1	26.5
Donor 7 PB	78	35.2	1.1	4.5	70.7	26.9	20.9
Donor 8 PB	79	42	4.4	73.1	13.4	38.2	30.2
Donor 9 PB	85.1	26.8	11.8	29.7	34.7	20.4	17.4
Donor 10 PB	93.1	33.7	3.3	31.3	46.7	27.4	25.5
Donor 11 PB	89.3	40.1	2.1	16.2	48.2	26.7	23.8
Donor 12 PB	89.3	40.1	3.6	28.6	39.8	28.9	25.8
Donor 13 PB	70.2	37.8	6.7	28.6	38.6	27.9	19.6
Donor 1 LF	91.3	25.2	6.7	25.9	45.9	19.8	18.06
Donor 2 LF	88	28.5	4.1	40.2	39.2	23.8	20.94
Donor 3 LF	90.3	36.6	0.15	0.48	74.8	27.6	24.93
Donor 4 LF	75.8	35.6	3.6	35.2	43.6	29.3	22.24
Donor 5 LF	78.1	47	2.4	46.2	35.9	39.7	31.02
Donor 6 LF	82	38	0.9	2.2	74.4	29.5	24.15
Donor 7 LF	85	39.5	1.4	1.7	72.3	29.8	25.32
Donor 8 LF	86	49.7	2.7	63.9	11.6	38.9	33.42
Donor 9 LF	87.7	26.9	3.97	37.9	41.5	22.4	19.67
Donor 10 LF	91.5	21.6	3.1	38.3	43.2	18.3	16.72
Donor 11 LF	95.1	36.6	5.01	24.1	44.9	27.1	25.76
Donor 12 LF	61.5	42.7	4.8	35.6	41.2	34.8	21.43
Donor 13 LF	68.1	60.2	0.06	0.12	58.7	35.4	24.14

The flow cytometry analysis results of the NK cells' subpopulation compositions, using CD3, CD16, and CD56 markers. (PB=Peripheral blood, LF=Leukocyte reduction filter, LYMPH=Lymphocytes)

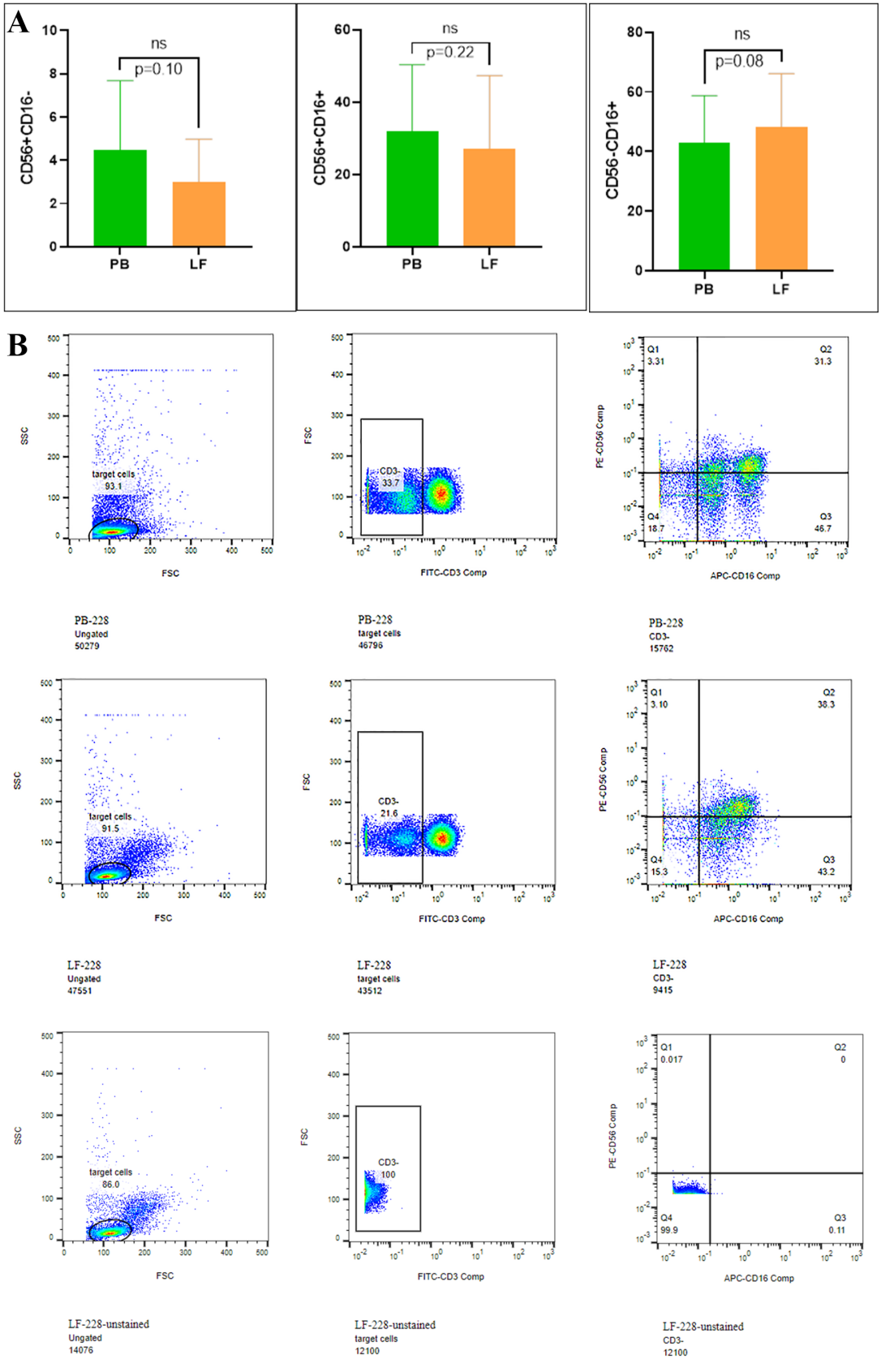

Figure 3. A) Statistical comparison of NK cells' major subpopulations in samples obtained from peripheral blood (PB) and used leukocyte-reduction filters (LF). The statistical significance was calculated by paired T-test. (N=13, *P<0.05, ns=not significant). B) An example of flow cytometry data analysis procedure of peripheral blood (PB) and used leukocyte-reduction filters (LF) NK cells by CD3, CD16, and CD56 staining.

cells from the leukocyte reduction filter does not disturb the natural ratio of the different NK subpopulations, and the filter-extracted NK cells could be considered a normal representation of the donor blood's NK cell composition.

MACS Purity

We used negative selection MACS to isolate the NK cells from the obtained sample. The subsequent flow cytometry analysis of CD56 and CD16 showed that high purity of the NK cells was achieved through this step. (87±4, data not shown).

Viability of the NK Cells Obtained from Leukocyte Reduction Filters (Flow Cytometry of 7-AAD)

We performed a standard viability test using the flow cytometry after the 7-AAD staining to examine this possibility. The results are shown in Table 3 and Figure 4.

Table 3. Flow cytometry analysis of the NK cells' Viability

Sample	%Viable cell (7-AAD Negative)	%Dead Cell (7-AAD positive)
Donor 1 LF	90.7	9.28
Donor 1PB	91	9.04
Donor 2 LF	86.4	13.6
Donor 2 PB	92	7.97
Donor 3 LF	88.6	11.4
Donor 3PB	89.8	10.2

Flow cytometry analysis results of the NK cells' viability using 7-AAD staining. (PB=Peripheral blood, LF=Leukocyte reduction filter)

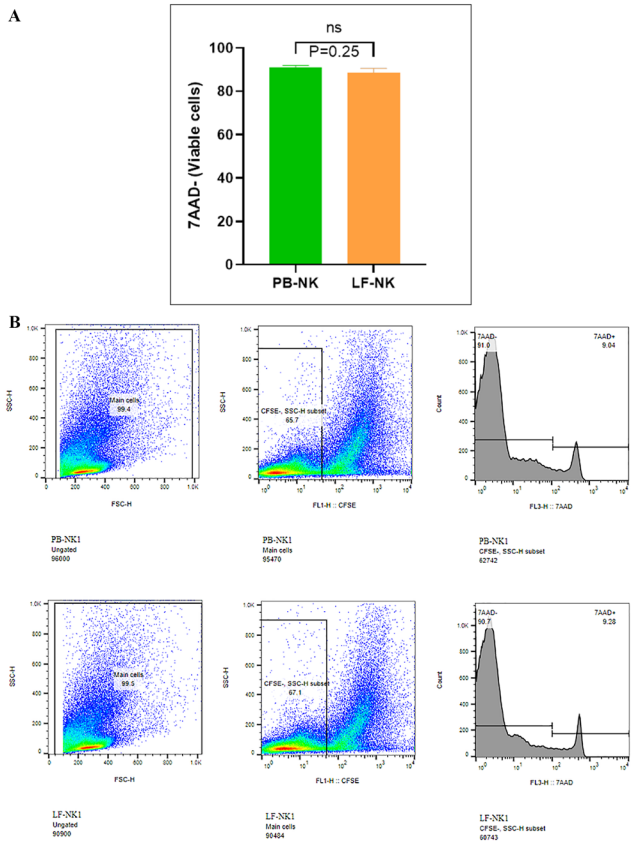

Figure 4. A) Statistical analysis of NK cells' viability differences between NK cells obtained from peripheral blood (PB) and used leukocyte-reduction filters (LF). The statistical significance was calculated by Wilcoxon signed-rank test (N=3, *P<0.05, ns=not significant). B) An example of flow cytometry data analysis procedure of 7-AAD staining of NK cells obtained from peripheral blood (PB) and used leukocyte-reduction filters (LF).

The paired analysis of our data indicates that there is no statistically significant decrease in the overall viability of the isolated NK cells. The average percentage of the viable NK cells was 88.6±2.2 for leukocyte reduction filter samples and 90.9±1.1 for peripheral blood samples. (N=3, P=0.25).

Functional Assays of the NK Cells Obtained from Leukocyte Reduction Filters

We proceeded with a 4-hour co-culture of the NK cells the with k562 cell line with the target: effector ratio of 1:4.

To compare the cytotoxic potency of the filter-obtained NK cells, we performed flow cytometry of CFSE, annexin V, and 7-AAD. CFSE staining of K562 cells before the co-culture was used as a gating control for the k562 cells in the flow cytometry analysis. We analyzed the results as paired and compensated the target cell line's spontaneous death using the k562 only wells as the control. The results are shown in Table 4 and Figure 5. The analysis rejects any statistically meaningful deterioration in the NK cells' cytotoxic potency in leukocyte reduction filter-extracted cells compared with the peripheral blood-obtained NK cells. (N=3, P=0.5)

Degranulation Capacity of the NK Cells Obtained from Leukocyte Reduction Filters (Flow Cytometry of CD107a)

We performed the flow cytometry analysis of CD107a surface marker expressions on three donors' samples after the co-

Table 4. Flow cytometry analysis of NK cells' cytotoxicity

Sample	%Early Apoptosis (AnexinV$^+$ 7AAD$^-$)	%Late Apoptosis (AnexinV+ 7AAD+)	%Necrosis (AnexinV- 7AAD+)	%Experimental Apoptosis	%Specific Apoptosis (Experimental -Spontaneous Apoptosis)
Donor 1 LF+K562	8.22	2.22	14.9	25.3	13.3
Donor 1 PB+K562	7.12	2.22	9.3	18.6	6.6
Donor 2 LF+K562	4.5	1.6	8.9	15	3
Donor 2 PB+K562	10	2.7	7.9	20.6	8.6
Donor 3 LF+K562	17.9	20.9	1.84	40.64	28.6
Donor 3 PB+K562	15.4	17.9	0.94	34.2	22.2
K562 (Control Group)	7.3	0.78	4	12	0

The flow cytometry analysis results of the NK cells' cytotoxicity against CFSE-stained k562, using 7-AAD and annexin-V. Experimental apoptosis indicates the observed death of K562 cells, calculated by the sum of early apoptosis, late apoptosis, and necrosis. Specific apoptosis is the adjusted value based on the K562 control. (PB=Peripheral blood, LF=Leukocyte reduction filter)

Table 5. Flow cytometry analysis of the NK cells' degranulation

Sample	%NK+K562	%CFSE$^+$(k562 cells)	%CFSE$^-$(NK cells)	%CD107a$^+$ In CFSE$^-$
Donor 1 LF+K562	99.1	15.6	84.2	89.9
Donor 1 PB+K562	98.7	25.8	73.5	89.2
Donor 2 LF+K562	98.9	23.3	76.6	91.1
Donor 2 PB+K562	98.7	23	76.8	92
Donor 3 LF+K562	98.1	76.5	23.2	89.6
Donor 3 PB+K562	98.5	78.3	21.5	94.2
NK/K562(CFSE) Gating Control	98	41.2	56.7	0.094

The flow cytometry analysis results of the NK cells' degranulation events against CFSE-stained k562, using CD107$^+$ marker. CFSE$^-$CD107$^+$ events are the NK cells that have degranulated. (PB=Peripheral blood, LF=Leukocyte reduction filter)

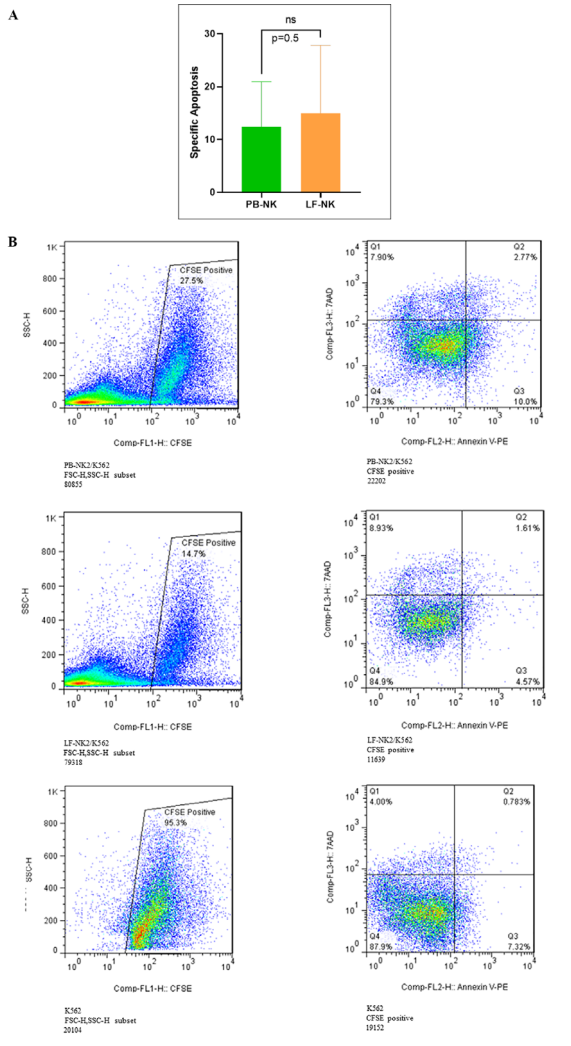

Figure 5. A) Statistical comparison of Cytotoxicity potency of NK cells obtained from peripheral blood (PB) and used leukocyte-reduction filters (LF) by flow cytometry of 7-AAD and annexin-V in k562 cells. Specific apoptosis was calculated by subtracting spontaneous apoptosis from experimental apoptosis. The statistical significance was calculated by Wilcoxon signed-rank test (N=3, *P<0.05, ns=not significant). B) An example of flow cytometry data analysis procedure of 7-AAD and annexin-V in k562 cells co-cultured with NK cell obtained from peripheral blood (PB) and used leukocyte-reduction filters (LF). The third row is the flow cytometry of k562 cell line only which served as the control.

culture with the K562 cell line. CD107a is an indicator of the NK cell activation and degranulation events in response to k562 tumoral cells. Analysis results shown in Table 5 and Figure 6, indicate that obtaining the NK cells from this source will not alter the CD107a expression patterns on the co-cultured NK cells (N=3, P=0.5); thus, the NK cells obtained from leukoreduction filters are comparable with the peripheral blood's NK cells regarding degranulation capacity.

DISCUSSION

Due to their pivotal role in immune system responses, the researchers' demands for the NK cell sources have increased substantially. Three studies in South Florida, Switzerland, and Singapore established a range of -respectively %4 to %18 [9], %5 to %30 [10], and %7 to %17 [11] of NK cells in the peripheral blood lymphocytes population of an adult person. This low abundance of NK among PBMCs and the fact that because of ethical concerns one can only donate a limited volume of blood for research purposes, has become one of the hindrances in the way of the NK-related research and medical developments.

To address this issue, the researchers have used a variety of strategies to increase the number of the available NK cells. To categorize, the first group of strategies is to ex vivo expand the NK cell from cord blood cells. Spanholtz et al. achieved this goal by developing NK cells from cord blood $CD34^+$ cells [4]. Similarly, Shah et al. incorporated $CD3^+$ depleted cord blood mononuclear cells to expand large quantities of NK cells [12]. Both methods are time-consuming (Spanholtz's method requires at least 35 days and Shah's method 14 days), and both need an array of cytokines, thus could be expensive.

The second group of strategies is similar to the first group in the sense that they consist of a series of differentiation steps to produce NK cells but differ mainly because the initial $CD34^+$ cells are not acquired from cord blood but rather from an adult donor's peripheral blood. This may be more convenient than collecting cord blood, but remains time-consuming and expensive. For example, the protocol devised by Yoon et al. takes 42 days

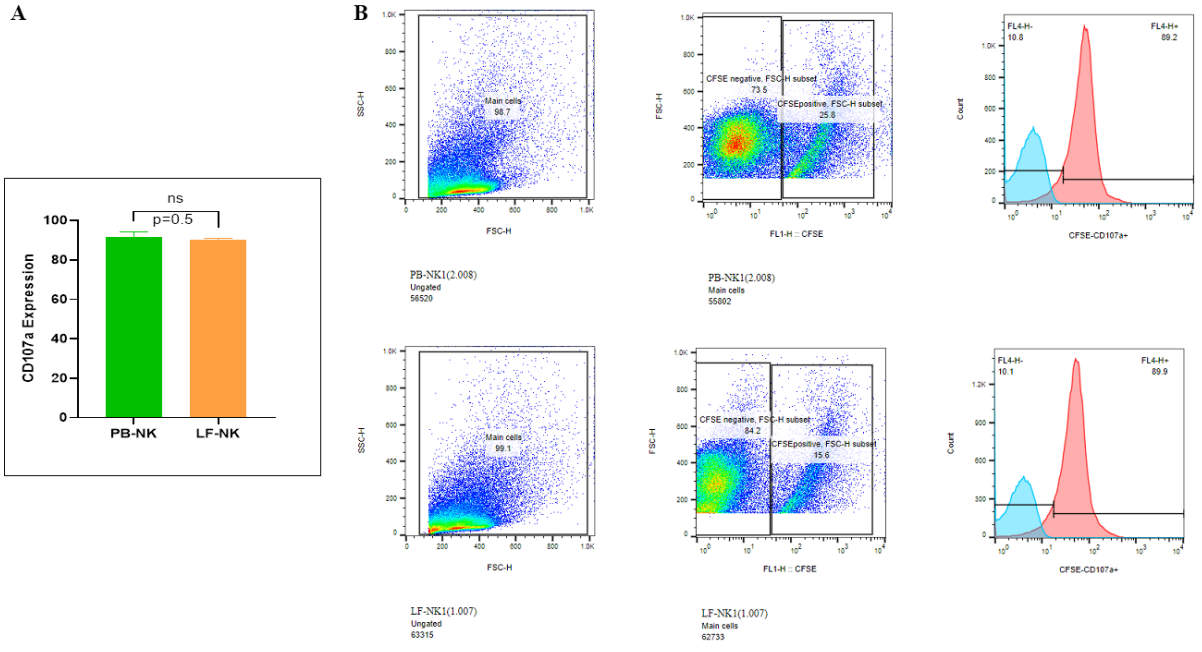

Figure 6. A) Statistical comparison of CD107a expression on NK cells obtained from peripheral blood (PB) and used leukocyte-reduction filters (LF) after 4-hours co-culture with K562 cells. The statistical significance was calculated by Wilcoxon signed-rank test (N=3, *P<0.05, ns=not significant). B) An example of flow cytometry data analysis procedure of peripheral blood (PB) and used leukocyte-reduction filters (LF) NK cells by CD107a staining (after 4-hours co-culture with k562)

and requires human stem cell factor (SCF), Flt3 ligand, Hydrocortisone, IL-7, IL-15, and IL-21 [13]. Luevano et al. reviewed the process of generating NK cells from hematopoietic stem cells in detail and particularly explored the cytokines used in such practices [14].

The third approach still requires a series of differentiation, but the starting point would be human embryonic stem cell lines. Eguizabal et al. reviewed this strategy in detail [15]. As an example of this approach, Woll et al. incorporated the H9 hESC line and developed NK cells through a series of differentiation steps. The problems mentioned above persist in this approach: it is time-consuming (47 to 55 days) and expensive because it requires co-culture and a variety of cytokines and reagents [16].

In the fourth strategy, researchers attempted to incorporate NK cell lines or establish new NK cell lines. To date, a multitude of NK cell lines (to name a few, YT [17], KHYG-1 [18], NKL [19], and NK-92 [3]) have been established. Currently, NK-92 is the most widely used NK cell line among clinical researchers. Although proven safe and superior in many clinical trials [20], NK-92 possesses certain characteristics which may make it not a precise equivalent of peripheral blood NK cells for research purposes: First, NK-92 is generally considered as a KIR^- cells [21], although this contributes to the superiority of NK-92 cytotoxicity, it may induce problems in research concerning the NK cells. Second, NK-92 cells lack surface expression of CD16 [3]; thus, for instance, they cannot be used in research concerning antibody-dependent cell-mediated cytotoxicity (ADCC).

The last strategy category is to expand mature $CD56^+CD3^-$ NK cells collected from peripheral blood. Numerous methods and protocols have been devised for this task which vary in detail and reviewing them are beyond the scope of this article; nevertheless, what they share is that they require cytokines/feeder layers, and the process takes several weeks. Another disadvantage that should be taken into account is that after several weeks of expansion, the produced NK cells show signs of exhaustion and senescence [22].

Nevertheless, a readily available and seemingly economical source for NK cells has been generally overlooked, namely, used leukocyte reduction filters. According to WHO, in 2018, roughly 118.4 blood units were donated worldwide. %60 of them were collected in middle-income or low-income countries [23]. Considering that pre-storage leukocyte reduction is now a mandatory procedure in transfusion medicine in most countries, and that according to our study, per every filtration of 450 ml of blood, on average 3.15E+09±1.07E+09 leukocytes are being entrapped in the used filters, the total number of blood's cellular component being discarded is immense. To add, because of the new practices in blood transfusion, one traditional source of PBMCs, buffy coats, is getting scarcer over the years. Thus, in the quest for a new source of blood components, used leukocyte reduction filters have been proposed by researchers.

The first paper we found proposing the extraction of leukocytes from used leukocyte reduction filters was by Longley and Stewart in 1989 [24]. Note that the U.S Food and Drug Administration (FDA), issued a memorandum in 1996, with the title "Recommendations and Licensure Requirements for leukocyte-Reduced Blood Products", recommending the usage of pre-storage leukocyte reduction [5]; thus, since the application of leukocyte reduction filters has yet to become universal at the time that Longley and Stewart published their paper, the topic was dropped. By the 2000s, a series of papers re-examined this suggestion. Successful recovery of B lymphocytes [8, 25, 26], T lymphocytes [8, 26, 27], $CD14^+$ monocytes [26, 28], Dendritic cells [8, 26, 29], NK cells [8, 26], $CD34^+$ cells [30-32], neutrophils [33] and peripheral blood-derived endothelial progenitor cells [34] have been reported by the researchers. The potential benefits of used leukocyte reduction filters are not limited to harvesting WBCs [35], as researchers extracted defensins [33, 36, 37] and large quantities of DNA [38] from used leukocyte reduction filters. Also, in one study, it has been confirmed that the B and T lymphocytes extracted from the leukocyte reduction filter could be successfully cryopreserved [39].

In agreement with the research reviewed above, our results also confirm the feasibility of extracting a high quantity of every major blood cellular population from Macopharma leukocyte reduction filters. All subsets of PBMCs (including lymphocytes encompassing NK cells) were present in high quantity. Our recovery rates (see Table 1) were close to other studies incorporating other leukocyte reduction filters. In accordance with Meyer et al. [8], changing the composition of the elution buffer did not have any significant impact on either the overall recovery rates or the population compositions of the recovered cells. This indicates the robustness of the used leukocyte reduction filters as a source of PBMCs.

As stated before, Meyer et al. and Izquierdo et al. have reported the presence of the NK cells among the leukocyte reduction filter-extracted PBMCs [8, 26]. To see if the NK cells available from used leukocyte reduction filters check criteria other than quantity, we have further explored these NK cells and confirmed that using filters as a source would not introduce new variables into one's study design.

Different WBC population compositions were observed after flushing the cells entrapped in used leukocyte reduction filters. The recovery rates of neutrophils and basophils are the lowest, while lymphocytes are the easiest WBC population to extract from the filters. Because the NK cells are found in the lymphocyte WBC population, this fact is favorable in our study. These recovery rates averages, however, may vary in leukocyte reduction filters by different manufacturers.

We then investigated the NK cells' subpopulation composition. As stated in the results section, every subpopulation of NK cells based on CD3, CD16, and CD56 can be recovered from leukocyte reduction filters with a proportion statistically not

different from the donor's peripheral blood. This confirms that using leukocyte reduction filters as the NK cells source will not lead to selective depletion or over-representation of any NK subpopulation, thus making the filters a reliable source for research in different NK cell subpopulations.

Compared with the direct isolation of the NK cells from peripheral blood samples, using filters as an NK source requires additional steps. These extra steps may impose chemical and mechanical stress on the NK cells and may lead to a marked decrease in the cell's viability and overall health. To examine this possibility, we conducted 7-AAD viability tests. Necrotic or apoptotic cells are marked positive in the flow cytometry after 7-AAD. As we have stated in the results section, the impact of the aforementioned additional steps on the NK cells' viability is trivial, thus confirming the suitability of leukocyte reduction filters as a source of the NK cells in this regard.

Leukocyte reduction filters depend on chemical and physical bonds between the filter's matrix and the cell's surface; thus, theoretically possible that in this process, some NK cells' activation receptors be engaged, leading to unwanted activation of the NK cells. This is why, because of the physical and chemical stresses imposed on the cells during the filtration and recovery process, some cells may undergo apoptosis and necrosis: therefore, making an environment full of activation signals for the NK cells. The opposite may also be true; the NK cells extracted from leukocyte reduction filters may lose their degranulation capacity. Thus, to examine these possibilities, we conducted a flow cytometry analysis against CD107a. This protein will transiently surface on the cell after a degranulation event. Our results indicate that there is no difference between the activation patterns of filter-extracted NK cells and their counterparts which have been directly collected from the peripheral blood.

Conforming all the aforementioned criteria, we then investigated the NK cell's primary function: to compare the cytotoxic capacity of filter-extracted NK cells and NK cells directly collected from the peripheral blood. Our results indicate that extracting the NK cells from leukocyte reduction filters does not negatively affect their cytotoxic potency. Note that in our setup, the NK cells were not activated or treated with any cytokines, and the co-culture period was 4 hrs.; nonetheless, there were no statistically significant differences between the observed cytotoxicity of leukocyte reduction filter-extracted NK cells and their counterpart, which was directly collected from the same donor's peripheral blood.

CONCLUSION

Used leukocyte reduction filters can be considered a cost-effective, easy to obtain, and robust source of abundant research-grade NK cells. To tackle the issue of low counts of the accessible NK cells, our method presents a simple and cost-effective alternative to the currently used methods. The NK cells obtained through our approach show no statistically significant differences from the peripheral blood's NK cells in terms of viability, sub-population composition, and cytotoxic capacities.

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