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The Direct Influence of Cytomegalovirus Lysate on the Natural Killer Cell Receptor Repertoire

Saeede Soleimanian[1], Ramin Yaghobi[1], Mohammad Hossein Karimi[1], Bita Geramizadeh[1], and Jamshid Roozbeh[2]

[1] Shiraz Transplant Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[2] Shiraz Nephro-Urology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

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ABSTRACT

Natural killer (NK) cells are essential for controlling certain viral infections, including cytomegalovirus (CMV). In particular, the importance of NK cells in the context of CMV infection is underscored by the adaptive capabilities of these cells. Evidence suggests that some viruses can directly interfere with NK cell compartments and their activation and lead to shape-shifting the NK cell receptor repertoire. Still, it remains unknown whether the CMV can interact with NK cells without intermediaries. Here, we examined whether the direct effects of CMV lysate alter phenotypical properties of NK cells.

To investigate this issue, NK cells were isolated from the blood of CMV seropositive healthy donors by negative magnetic separation. Isolated NK cells were cultured in the presence of CMV lysate and analyzed for the expression of NKG2A, NKG2C, and CD57 by FACS caliber.

The results showed that NKG2C expression is significantly upregulated in the presence of CMV lysate compared to without stimulated group (mean increase, 6.65 %; 95% CI, 0.2582 to 13.02; p=0.043; R square: 0.38). Likewise, results have shown a significant decrease in the frequency of NKG2A+CD57- NK cell subsets (p=0.005; 95% CI, -13.49 to -3.151; R square: 0.5957) in the stimulated group compared to without stimulated ones.

According to these results, CMV may drive a direct influence on NK cell receptor repertoire, including the expansion of NK cells expressing NKG2C receptor, which is needed for further studies.

Keywords: Cytomegalovirus; Natural killer cells; NK cell lectin-like receptor subfamily

INTRODUCTION

Despite advances in cytomegalovirus (CMV) treatment, this infection still remains an important cause of morbidity and mortality in immunocompromised patients.[1] The full spectrum of CMV complications is still determined to be understood.[2,3] Therefore, assessing immunity status and specific immune responses against CMV infection in different aspects could be a clue to better understanding the efficient therapeutic strategies.[4,5] Natural killer (NK) cells as immune invigilators are promising tools to tackle viruses, identify and attack virus-infected cells without being sensitized to antigens.[6-9] They respond to

Corresponding Author: Ramin Yaghobi, PhD;
Shiraz Transplant Research Center, Shiraz University of Medical Sciences, Shiraz, Iran. Tel: (+98 71) 3628 1529, Fax: (+98 71) 3628 1529; E-mail: rayaviro@yahoo.com

the herpes family, specifically CMV infection, and their response is essential for immunosurveillance in these infections.[10-12] Accumulating evidence suggested the intriguing role of NK cells in the fight against CMV infection.[13,14] Based on the current knowledge, the antigen-specific phenomenon of NK cell memory to CMV infection leads to a long-lasting expansion of educated NK cells.[15] In the course of virus-host coevolution, CMV has acquired many abilities to maintain viral fitness. NK cells are regulated by an array of inhibitory and activating surface receptors. Despite lacking antigen-specific rearranged receptors in NK cells, NK cell development is rapidly driven toward a mature stage of differentiation characterized by a CD56dim NKG2A−NKG2C+CD57+ NK cell mature phenotype in individuals experiencing CMV reactivation.[16-18]

Notably, several studies have shown that CMV UL40-derived peptides can interact with Natural killer group (NKG) 2A (as an inhibitory receptor) and NKG2C (as an activating receptor) of NK cells through upregulation of HLA-E. Adaptive NKG2C+ NK cells selectively recognize CMV strains through HLA-E-binding to UL40-derived peptide that triggers expansion of adaptive NK cells.[19] NKG2A has presented a higher affinity for HLA-E, which tends to compete with NKG2C for ligand engagement.[20] However, there is considerable heterogeneity among adaptive NK cells, and distinct peptides can drive diversity in adaptive NK cell populations. Still, it should be identified whether NKG2C can recognize UL40 peptides during CMV infection and if the activation of NKG2C+ NK cells is due to such recognition.[21-23] Based on the evidence, the mechanism by which NK cells are activated has not been precisely elucidated. In addition to recognizing different herpes viruses through several activating receptors and co-receptors and killing them via antibody-mediated NK cell cytotoxicity (ADCC), the direct activation of NK cells via Toll-like receptors (TLRs) has been identified. NK cells possess different functional TLRs that allow them to sense and respond against herpes viruses by recognizing their pathogen-associated molecular patterns (PAMPs).[24,25] For instance, the expression of pro-inflammatory cytokines is stimulated through TLR3 and TLR9 molecules in CMV infection.[26] Hence, NK cells could be activated by encountering herpes viruses in the absence of antigen-presenting cells (APCs) or accessory cells. Indeed, NK cells recognize through TLRs and drive the response,[27] contrary to the notion that the presence of accessory cells, including dendritic cells and monocytes/ macrophages, are required to induce NK cells to function via pathogens.[28] Therefore, it remains unclear whether adaptive features of NK cells could be established directly through sensing inflammation of CMV. Notably, former investigation on NK cells has made us realize that they have vast functional capability, including APC- like features of NK cells in regulating T-cell activation.[29] To evaluate this ability and gain further insight into how these cells undergo direct interaction with CMV, immunophenotypic analysis concerning distinct immune biomarkers study affected by CMV lysate (a mix of CMV antigens from all stages) would be appreciated. This study could be valuable for exploring the phenotypic changes of untouched NK cells with virus lysate, without the impact of other immune cells on NK-cell activation through cell-cell interactions or by secretory molecules. This study was conducted to assess the effect of CMV lysate on the maturity and activation of NK cells.

MATERIALS AND METHODS

Subjects and Sample Processing

In this study, 20 mL, ethylenediaminetetraacetic acid (EDTA)-treated blood samples were taken from 10 CMV seropositive subjects without underlying diseases (70% male, age mean; 40). The Ethics Committee of Shiraz University of Medical Sciences approved the study (IR.SUMS.REC. 1396.S289). All participants provided written informed consent at study entry.

Determination of Anti-CMV IgG Titers

The plasma of samples was collected to determine CMV serostatus by titers of IgG antibodies specific for CMV using a Human Anti-Cytomegalovirus IgG ELISA Kit (DIA.PRO, Milano, Italy), following the manufacturer's instructions.

NK Cell Isolation and NK Cell Culture

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy blood donors by Ficoll Paque (Lymphodex. Inno-Train-Spain) density gradient centrifugation and cryopreserved until processed. NK cells were isolated from PBMCs using commercial magnetic bead separation assays (NK Cell Isolation Kit, Miltenyi Biotec, Bergisch Gladbach, Germany) to

acquire highly purified NK cell subsets. PBMCs were thawed and left in a culture medium for 2 h before each experiment. The culture medium was RPMI 1640 (Gibco Laborato- ties, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Gibco Laboratories), L-glutamine (2 mM), and 1% antibiotic mixture (penicillin-streptomycin 5 mg/mL). NK cells were isolated from PBMCs through negative selection using an NK Cell Isolation Kit I (Miltenyi Biotec, Bergisch Gladbach, Germany). The CD3−CD56dim and CD3−CD56 bright NK cell populations were obtained according to the expression of CD56 and lack of CD3 surface marker.

Stimulation of Isolated NK Cells with CMV Cell Lysate

Cytomegalovirus cell lysate antigen (CMV-CL-100) was commercially obtained from Native Antigen, Oxford, UK (Batch No: 171720120) that is a mix from all stages of its replication life cycle, which be produced from AD-169 strain in Human Foreskin Fibroblasts (HFF). In the absence of stimulation via cytokine, we tried to determine the activation of NK cells through changes in the expression of their receptors after CMV lysate exposure. NK cells cultured in the presence of CMV lysates with 1.25, 2, 3, and 5 µg/mL of CMV virus lysate (CMV purified virus lysate; Native Antigen) and stimulation with CMV lysates was compared to NK cells cultured without CMV lysates (0 µg/mL) for the same time intervals as a negative control. The mock condition was investigated without lysate called without stimulation (w.i.). Furthermore, 2×10^5 isolated NK cells from 10 CMV-seropositive donors were incubated at 5% CO2 and 37°C, in a humidified atmosphere at different time intervals; 2, 4, 6 hours overnight, to determine the optimum concentration for stimulation. Then NK cells were plated at a concentration of 2×10^5 cells per well in a 96-well plate containing either CMV lysate. To induce in vitro isolated NK cells, 2×10^5 NK cells were cultured in 96-well round-bottom plates (Costar). After each time point, cells were collected, and harvested cells were stained with anti-CD3- PerCP-Cy5.5, CD56-PE, NKG2C-Vio-Bright, CD57-PerCP-Cy5.5, and NKG2A-PE to analyze. First of all, the viability of isolated NK cells was assessed with a combination of Annexin V-FITC staining and a membrane-impermeable dye propidium iodide (PI) (Biolegend-USA) and analyzed by a one laser flow cytometry system; using a 488 nm laser.

Flow Cytometric Analysis to Define the Purity of Isolated Samples

The purified fraction was stained with anti-human mAb specific for CD56 (to identify the proportion of purified NK cells in the lymphocyte population, i.e., "NK-cell purity") and anti-human mAb specific for CD3 (to determine the percentage of T cells, i.e., "T cells Contamination"). Forward scatter and side scatter are used to assess the differentiation of cell debris from lymphocytes.

Antibodies and Immune Fluorescent Staining

Purified NK cells were stimulated with the CMV lysate strain AD169 at different concentrations (1.25, 2, or 3 µg/mL) at three-time intervals (2, 4, 6, and 24 hours).

After NK cell isolation and evaluating purity, we used the activating and inhibitory marker (NKG2C and NKG2A, respectively) and maturation marker (CD57), which facilitates the discrimination between adaptive NK cells[30,31], and conventional ones. Isolated NK cells were stained with the following panel of mouse anti-human mAbs: antiCD3-PERCPCY5.5 (clone UCHT1, Biolegend, USA), anti-CD56 PE (clone: HCD56, Biolegend, USA) for the evaluation of purity, and NKG2A-PE (clone: REA110, Miltenyi Biotec, Bergisch Gladbach, Germany), NKG2C-Viobright FITC (clone: REA205, Miltenyi Biotec, Bergisch Gladbach, Germany), and anti-CD57 PERCPCY5.5 (clone HNK-1, Biolegend, USA) for phenotypical assessment of NK cells were used. The antibody solution was added to cell suspension followed by incubation in the dark at 4°C for 20 min. Titration of all antibodies was performed to gain the optimal working concentrations. Blocking was done for unspecific binding by pre-incubation with 10% heat-inactivated bovine serum. Unstained controls and fluorescence minus one controls (FMO) were utilized to set compensation controls of spectral overlaps, establishe positive/negative borders, and prove nonspecific binding. It is needed to use FMO to select the proper threshold of the stained cells with anti CD57–PERCPCY5.5 antibodies. Isotype controls were used to account for the nonspecific antibody binding, including; (Iso IgG1, k –PE, IgG1, k –PERCPCY5.5,

IgM1, k –PERCPCY5.5, Biolegend, and REA control 9 (REA205), Miltenyi Biotec). Cells were suspended in a staining buffer, and data acquisition for 20000 events was performed using the BD FACS caliber instrument. Data acquisition and analysis were made using FlowJo (version 10; Tree Star).

Statistical Analysis

Statistical analyses were performed using Paired t-test or two-tailed Student t-tests (paired), depending on the bivariate normality assumption according to the D'Agostino & Pearson omnibus normality test. The statistical tests were performed using GraphPad Prism 7.0 (GraphPad Software, USA). The data were represented as the mean ± SEM. Significant differences between groups are represented by *p<0.05, **p<0.01, and ***p<0.001.

RESULTS

Analysis of CD56+ NKG2C+NK Cells Expressing NKG2A and CD57 upon Stimulation with CMV Lysate

In the present study, NK cells were purified from healthy CMV-seropositive donors. Annexin–PI staining demonstrated the high viability at the steady-state of 95% (cell viability was not affected by the CMV lysate treatment, no changes in annexin V binding and PI uptake of the treated NK cells were observed). In addition, staining with PE-conjugated anti-CD56 and PERCPCY5.5-conjugated anti-CD3 antibody was done to identify NK cells and T cells, respectively.

NK cells respond to CMV infection by expanding an adaptive NK cell population, best characterized by the expansion of NKG2C+ CD57+NKG2A− NK cells. The gating strategy employed identifies the NK cell subset expressing NKG2C, CD57, and the lack of NKG2A receptor.

As shown in Figure 1, we set the most effective concentration of virus at three-time intervals including 2, 6, and 24-hours post-stimulation (Figure1-A), and the expression of NKG2C, NKG2A, as well as CD57 cell surface receptor, was determined. It should be mentioned that as no significant difference was found between 4 and 6 h in our setup process, hence the 4h period was removed from our conditions. The concentration of 3 µg/mL was found the optimum, whereas 5 µg/mL was inhibitory for cells, and induction through 1.25 and 2 µg/mL concentrations did not increase. As demonstrated in Figure 1, using 3 µg/ml virus lysate at 24 h time point indicated the most induction through increased expression of the NKG2C NKG2A CD57 NK cell subset. Therefore, a final concentration of 3 µg/mL for CMV lysate at the peak time of production (24 h) was set in this assay. It should be noted; we have done a killing assay through the investigation of the frequency of CD107a and granzyme B. Still, we did not observe a significant difference between lysate and without lysate stimulation with this sample size, and our experiment will be continuing with more sample size.

As displayed in Figure1-B, for a representative donor, only about 10% of the CD57+NKG2A-NK cells were NKG2C positive cells in CMV-free cultures. Still, then after direct exposure to virus lysate, the increased expression of NKG2C receptors in the CD57+NKG2A- NK cell population was detected. For instance, in the representative donor, the first expression detectable with 3 µg/mL concentration of lysate at 2 and 6 –hours were 12.6% and 5.6%, respectively. Nevertheless, the maximum frequency was reached at 24-h post-infection with 71.1% (Figure 1B).

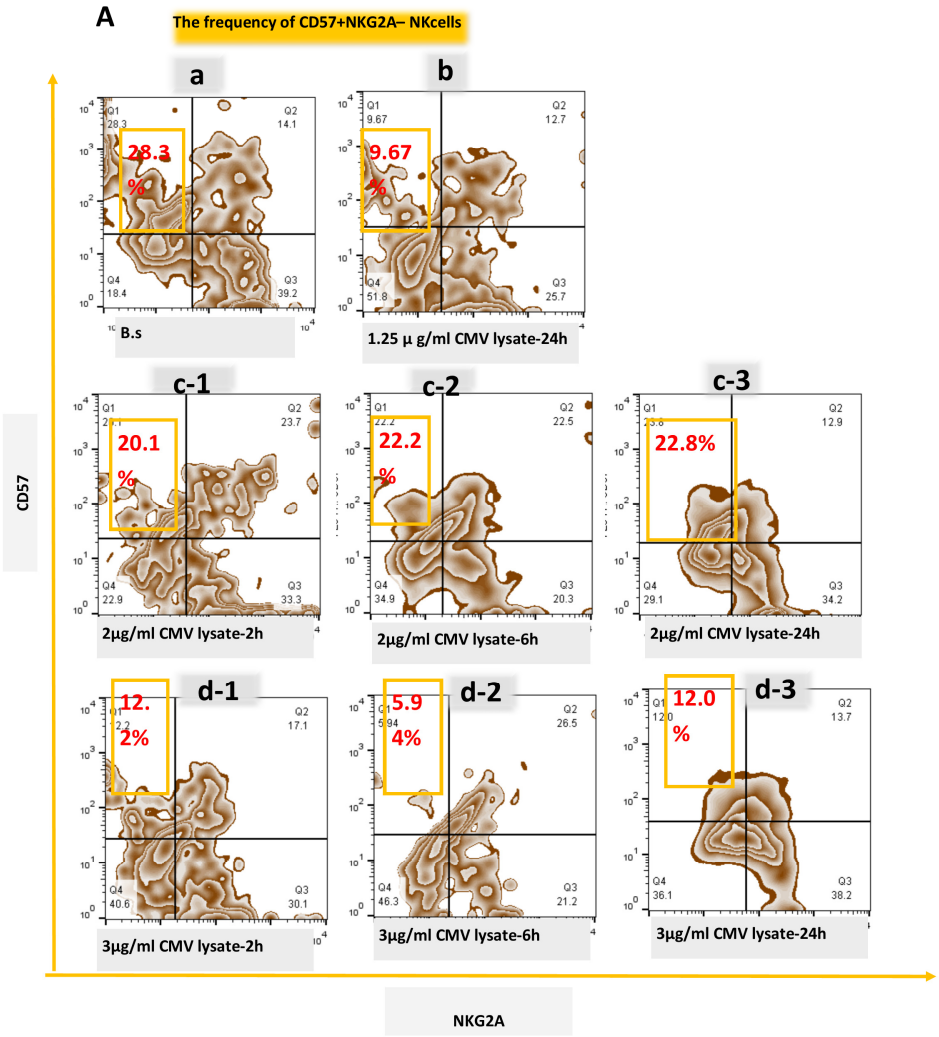
Significant changes in the expression of NKG2C+ NK cells were revealed in response to CMV lysate. The graphs in Figure 2A indicate that the NKG2C+ NK cell subset is significantly upregulated in the presence of CMV lysate compared to the control group without stimulation (mean diff; 6.65 %; 95% CI, 0.2582 to 13.02; p=0.043; R square: 0.38). A slight and non-significant decrease was also observed in the NKG2A expressing fraction (mean diff; 6.4%; 95% CI, -0.3697 to 13.19; p=0.19). But, total CD57 expression demonstrated non-significant difference (mean diff; 3.83%; 95% CI, -22.01 to 14.34; p=0.6443). As shown in Figure 2B, only a small fraction of the purified NK cells of control samples expressed NKG2C activation. Still, the presence of CMV lysate induced the cells to upregulate NKG2C expression. Indeed, data presented in Figure 2 indicates that CMV lysate could not present a significant effect on the NKG2A and CD57 production levels (Figure 2B). Still, CMV-induced activation of NK cells resulted in increased production of NKG2C receptors in the NK cell population. Therefore, CMV lysate at all concentrations of our study increased NKG2C+NK cells (Figure 2A and B).

The Frequency of CD57 Expression by Different Concentrations of CMV Lysate

According to our investigation, the expression of CD57 marker at different time points after exposure with CMV lysate was changed. For instance, in the representative donor in Figure 3, during early time points (2 h and 6 h), the frequency of the CD57 marker was upregulated; 44.5% and 37%, respectively. Whereas dropped expression was detected at late time point 28.7% (>16 h after stimulation), however, there were not any significant changes compared to without stimulation (Figure 3).

Evaluation of the NKG2A+ CD57- NK Cell Subset after Stimulation

Phenotypical analyses of NKG2A positive cells and CD57 negative cells after CMV stimulation were also performed on CD56+CD3- cells (see Figures 4-A and B). In parallel with increased NKG2C+NKcells, a significant decrease of NKG2A+CD57- NK cell population was observed. Results in Figure 4-C have shown a significant reduction in the NKG2A+CD57- NK cell subsets (p=0.005; 95% CI, mean of difference; 8.32±2.28; R square: 0.59).



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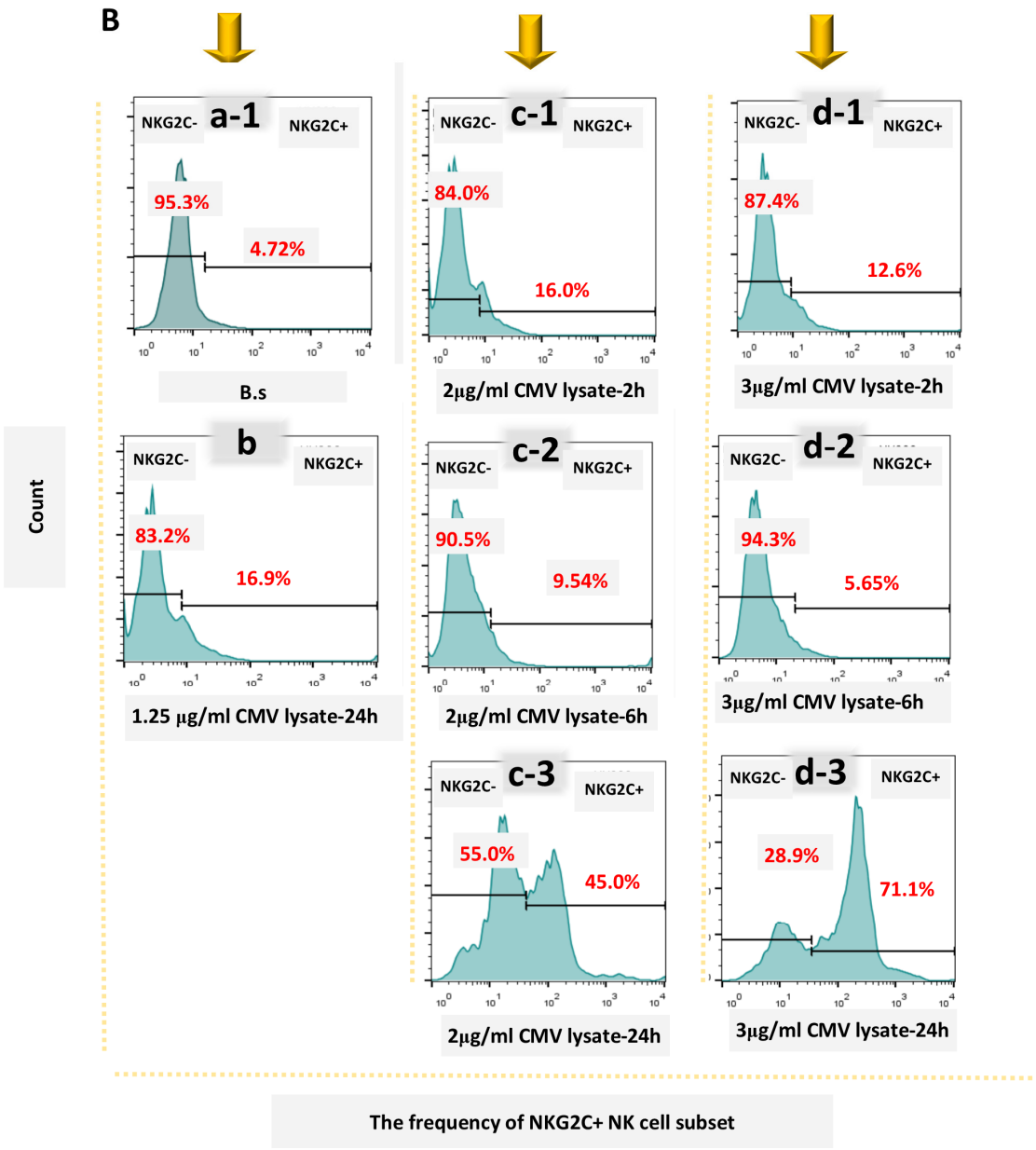

Figure 1. A: The frequency of NKG2C+ natural killer (NK) cells before and after stimulation with cytomegalovirus (CMV) lysate concentration: 1.25 µg/mL, µg/mL, and 3 µg/mL at three interval times; 2, 6, and 24 hours. w.s.; without stimulation. p.s.; -post-stimulation. B: Histograms of the frequency of NKG2C+ NK cell (gating on CD57+NKG2A- NK cells) without and with CMV lysate stimulation.

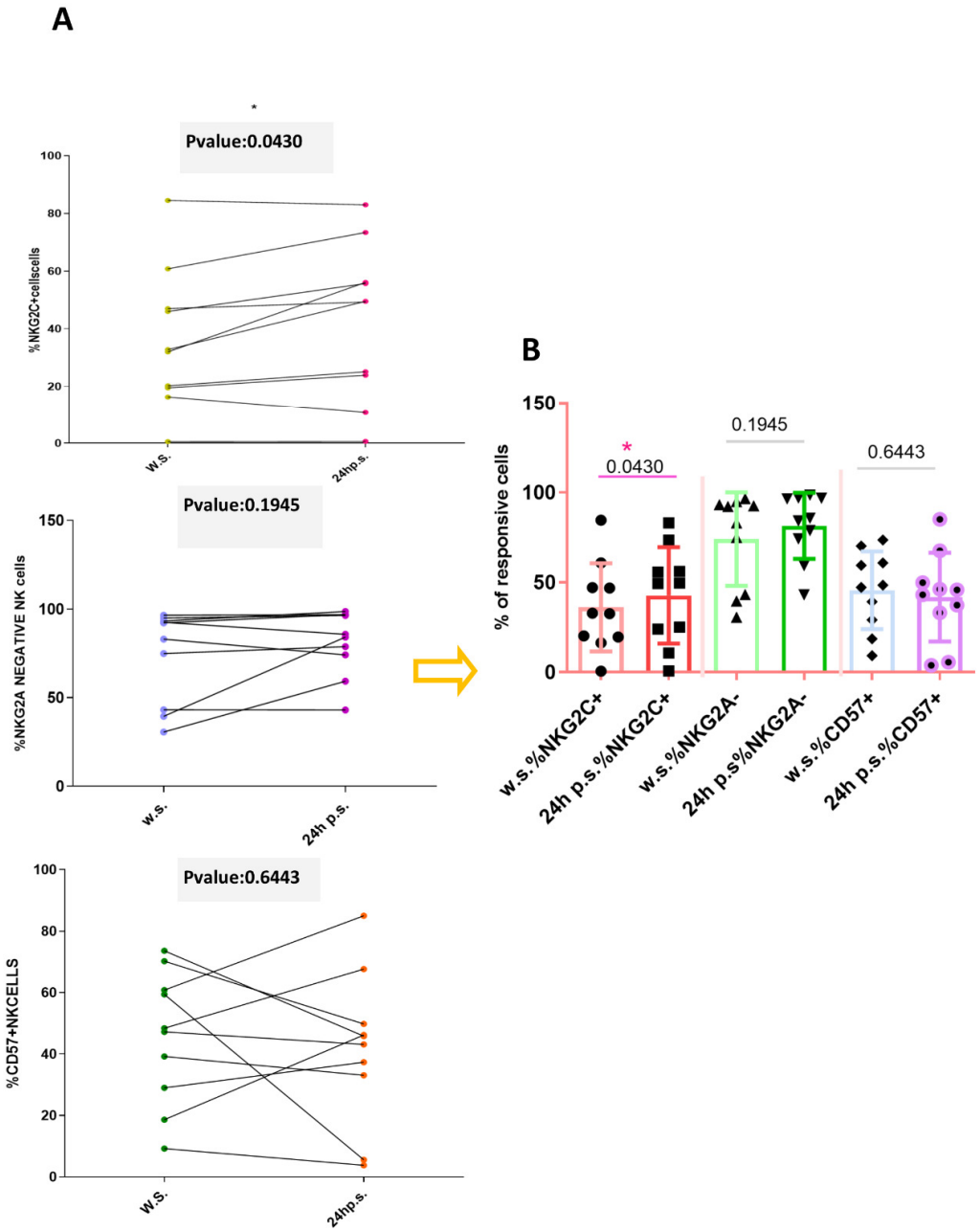

Figure 2. The graphs show the frequency of NKG2C+ natural killer (NK) cells, CD57+ NK cells, and NKG2A- NK cells without and with stimulation with cytomegalovirus (CMV) lysate. B. The frequency of all NK cell subsets in one graph. w.s.; without stimulation. p.s.; -post-stimulation. For each bar, data were presented as mean±SEM; n=10 in each group. Significant results: *p<0.05

NKG2A is the first inhibitory receptor to be changed during in vitro (and in vivo) NK cell differentiation. Indeed, concomitantly to NKG2C acquisition, NK cells down-regulate NKG2A receptors. In addition, our result indicated that in CMV- induced NK cells, partial down-regulation of CD57 occurred (expressed at lower levels than in NK cells without stimulation with CMV lysate).

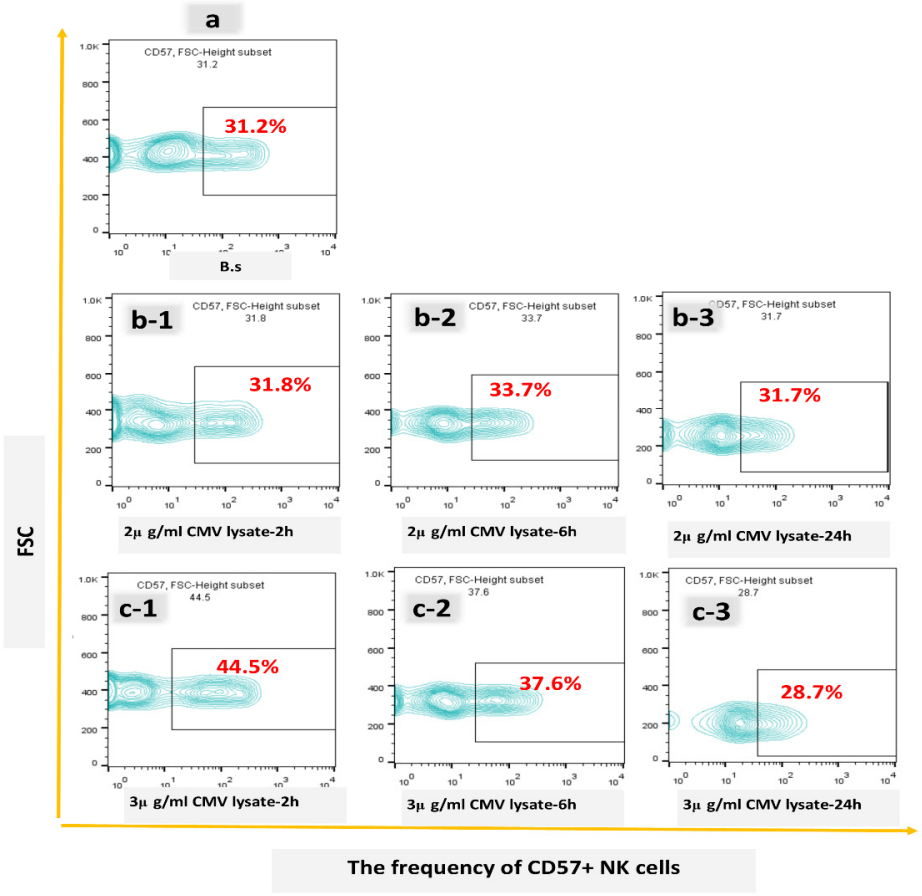

Figure 3. Data are representative of the mean frequency of CD57 expression by different concentrations of cytomegalovirus (CMV) lysate at three-time interval experiments. (a); The frequency of CD57+ natural killer (NK) cells before stimulation with CMV lysate. (b-1, 2 and 3); The frequency of CD57+ NK cells after stimulation with 2 µg/mL at 2, 6, and 24 hours, respectively. (c1, 2, and 3); The frequency of CD57+NK cells after stimulation with 3 µg/mL at 2, 6, and 24 hours, respectively. w.s.; without stimulation.p.s.; post-stimulation.

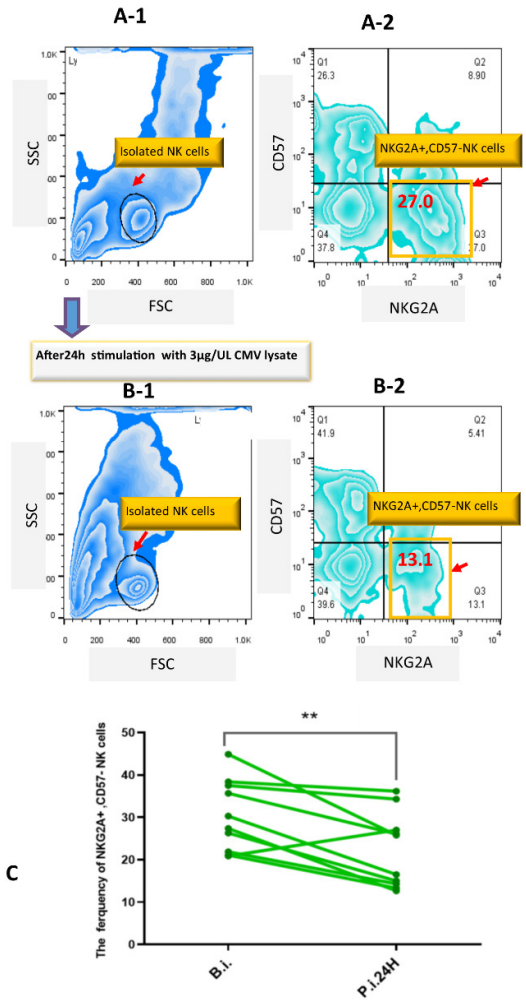

Figure 4. Analysis of the NKG2A+ CD57- natural killer (NK) cell subset after stimulation. (A-1, B-1); Forward scatter (FSC) versus side scatter (SSC) dot plot of isolated NK cells before and after stimulation with cytomegalovirus (CMV) lysate. (A-2, B-2); Gating isolated NK cells based on CD57 and NKG2A receptor and assessing the expansion of NKG2A+ CD57- NK cells before stimulation with CMV lysate. (C); The graph has presented the mean of difference±SEM before and after stimulation in NKG2A-CD57+ NK cells; n=10 in each group. Significant results: **p<0.01. The data are the means±SEM. w.s.; without stimulation.p.s.; post-stimulation

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DISCUSSION

Recent studies have revealed "memory-like" features for NK cells in which CMV infection has induced the conversion of conventional NK cells (NK convs) into adaptive NK cells (NK memory) in different inflammatory contexts.[32-34] Braud and colleagues found that NK cells expressing NKG2C have indicated long-term persistence and increased functional responsiveness. Primary CMV infection increases the capacity of NK cells for cytokine induction, which is maintained long after viral clearance. Subsequent exposure to CMV further elevates this capacity, suggesting that NKG2C+ NK cells retain their activity.[35] In several models, recurrent stimulation of NK cells leads to a more rapid and effective response.[36,37] These NK cells (NKG2C+, CD57+, NKG2A- subset) are preferentially expanded during acute CMV infection in the context of transplantation.[32,38] Notably, there are numerous modes that NK cells recognize the viral antigens and peptides.[21,39] Since the functional repertoire of NK cells is far more diverse than has been previously realized, we used two different approaches to evaluate the NK cell response to CMV lysate. First, to identify the target of responsive NK cells with adaptive phenotype, consisting of many CMV proteins, likely including PP150, PP65, UL16, UL18, and UL40.[21,40,41] These pools provide identification of individual target lysate. This approach was also suitable for determining whether an individual did or did not have an adaptive NK cell response to CMV lysate (without considering the specific CMV antigen and the extent of this cell response). Second, to define the frequencies of NK cells (with adaptive phenotype) reaction to direct exposure without needing accessory cells such as DC cells. In our study, the phenotype of adaptive human NK cell clones obtained by the virus lysate stimulates healthy individuals, determined by NKG2C, CD57, and NKG2A expression levels. Stimulation of NK cells via CMV lysate is associated with slight changes in the expression of NK cell receptors, including the upregulation of the NKG2C receptor, which has confirmed the reshaping of NK cells receptor repertoire. By the way, there are contradictory results regarding CD57 expression on NK cells during stimulation. We observed that CD57 expression at different time points after stimulation was altered during the time course of stimulation. At earlier time points (2 h and 6 h), it was upregulated, whereas dropped expression was detected at the late time point (>16 h after stimulation); however, there were not any significant changes compared to without stimulation. In 2018, Streltsova et al,[42] reported that, surprisingly, NK cells could acquire less mature phenotypes according to CD57 and NKG2A expression levels. They have shown that in specific conditions, CD57+ human NK cells can obtain the CD57negative phenotype and return to the previous maturation stage. Therefore, the loss of CD57 expression in the mature NK cells is fundamentally possible. Following this concept, it is suggested that reversibility of CD57 expression in our results might be due to late time points and chronic inflammation that lead to phenotype-shifting of the NK cells to the previous step in the particular condition. Moreover, Kobyzeva et al. reported that[43] since NK cells have dynamic plasticity in the pathological states, we couldn't ignore this concept that they can acquire de novo or lose their receptor expression.[44] In addition, we found that the NKG2A-NK cell population was decreased, although it is not significant statistically. NKG2A regulation may be linked to the loss (at least partial) of surface CD57 expression. Notably, other immune effectors, including T cells, could not influence NK cells activity because functional analyses of purified NK cells rather than PBMC were performed. Hence, in this condition, the antigen-presenting cells are absent. Notably, NK cells are important immune cells that support and drive the subsequent maturation of adaptive immunity subsets, particularly T cells.[45] Interestingly, evidence has shown that, as well as a response against virus-infected cells and tumor cells, a striking feature of these cells could be the APC-like properties to present antigen to CD4+ T cells. This new observation suggests a novel APC-like activating function for human NK cells, called NK-DC -like cells.[29] Notably, it is demonstrated that NK cells express MHC class II and TCR costimulatory molecules in vivo. Thus, APC-like NK cells can cross-talk with neighboring cells, including CD4+ T cells, and directly shape adaptive responses.[46] Furthermore, it has been revealed that cytotoxic T lymphocytes (CTLs) similar to NK cells have indicated APC features. With an antigen, self-presentation function might induce and multiply in vivo after exposure to microbial or viral peptides.[47] Considerably, the advantage of the APC

feature of NK cells might occur in the interaction of their receptor receptors with the MHC ligands of neighboring NK cells resulting in the development of the KIR+ NK cells from the HLA-DR neg NK cell subset.[42] Hence, this perspective of NK cells, besides the newfound understanding of NK cells' long levity feature, suggests this subpopulation as a potential target to augment immune response with higher effector functions and pathogen-specific protective qualities like those protected by memory lymphocytes.[48] In addition, growing evidence suggests that several viruses, including herpes virus, respiratory syncytial virus, and human immunodeficiency virus, can directly interfere with NK cell activity and induce changes in the NK cell receptor repertoire.[49,50] NK cells can appear as target cells for viral infections. Viruses have evolved multiple mechanisms to enter NK cells, alter the cellular effector function and disturb the subsequent immune response.[51] On the other hand, NK cells can adapt to the environment and exhibit significant functional plasticity,[52] including adaptive capabilities among its subpopulations.[53] This concept raises the possibility of more unexpected functional specialization and diversity than previously realized. The present work supports the growing idea that CMV lysate can shape NK cells. Hence, assessing the NK cell's properties in the independent APC status supports the growing number of reports challenging the belief that NK cells are invariable cells with stable features.

CMV infection drives modification in NK cell maturation, with a changed distribution of maturational subsets. In this work, an unexpected NK cell activation after induction with virus lysate is presented. It suggests that CMV lysate can drive modification in conventional NK cells in which the expansion of NK cells carrying NKG2C receptors occurs. A variety of simulation approaches based on cytokine treatment are used to activate and expand adaptive NK cells. Our results might be a small contribution to understanding NK cell response against CMV infection better. In this respect, this fundamental activation of NK cells contributes to the growing idea of NK cell plasticity, which is needed further study to strengthen NK cells utilization in therapeutic strategies.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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