СКООРДИНИРОВАННАЯ ЭКСПРЕССИЯ МАРКЕРОВ NK-КЛЕТОК И ОТВЕТА IgG ПРИ ИНФЕКЦИИ hCMV

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Резюме. Цитомегаловирус человека (hCMV) является распространенным вирусом, поражающим большую часть населения во всем мире. Естественные клетки-киллеры (NK) представляют собой иммунные клетки, которые играют решающую роль в борьбе с инфекцией hCMV. Несмотря на широкое распространение hCMV-инфекции, данных о взаимосвязи врожденного и адаптивного иммунитета до сих пор недостаточно. В этом исследовании изучалась взаимосвязь между экспрессией NK-клеточных маркеров и гуморальным иммунитетом во время инфекции hCMV. Было проанализировано 33 образца, полученных от здоровых волонтеров. Титр anti-CMV IgG антител измерялся в образцах сыворотки крови, а экспрессия NKG2C, HLA-DR, CD57, KIR2DL2/DL3 и KIR2DL1 на поверхности NK-клеток ($CD56^+CD3^-$) исследовалась в образцах PBMC методом проточной цитометрии. Для анализа процентного содержания различных субпопуляций NK-клеток в зависимости от титра IgG предварительно была проведена кластеризация всех полученных данных, по результатам которой было выделено 4 основных кластера. Выделенные кластеры продемонстрировали зависимость от уровня антител к hCMV, по которой были сгруппированы кластеры, соответствующие серонегативным и низко положительным образцам. Исследование показало, что инфицирование hCMV приводит к увеличению популяций NK-клеток, экспрессирующих маркер NKG2C, что коррелирует с более высокими уровнями ответа IgG на hCMV. Интересно, что мы выявили повышение $HLA\text{-}DR^+$ и снижение $KIR2DL1^+$NK-клеток со средним уровнем титра IgG к hCMV по сравнению с образцами, полученными от серонегативных и низко положительных доноров. Кроме того, была обнаружена статистически значимая отрицательная корреляция между NK-клетками $KIR2DL1^+$ и титром анти-hCMV IgG антител, в то время как положительная корреляция между HLA-DR и уровнем антител была отмечена только без кластера, соответствующего высокому уровню анти-hCMV IgG. Однако в данном исследовании не было обнаружено связи между экспрессией KIR2DL3 и CD57 на NK-

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клетках и уровнями IgG-ответа на hCMV инфекцию. Это указывает на то, что разные субпопуляции NK-клеток могут выполнять различные роли в регуляции гуморального иммунитета к hCMV. В целом результаты этого исследования дают ценную информацию о координации экспрессии маркеров NK-клеток и ответа IgG при инфекции hCMV.

Ключевые слова: NK-клетки, hCMV, IgG, NKG2C, HLA-DR, CD57, KIR2DL2/DL2, KIR2DL1, кластерный анализ, корреляционный анализ

COORDINATION OF NK CELL MARKER EXPRESSION AND IgG RESPONSE IN hCMV INFECTION

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Abstract. Human cytomegalovirus (hCMV) is a prevalent virus that affects a large proportion of the population worldwide. Natural Killer (NK) cells are essential immune cells that play a crucial role in controlling hCMV infection. Despite the wide spread of hCMV infection, there is still not enough data related to the association between innate and adaptive immunity. This study investigated the coordination between some of the NK cell markers expression and humoral immune response during hCMV infection. Thirty-three samples obtained from different healthy donors were investigated. The anti-hCMV IgG antibody titer was measured in serum samples, and expression of NKG2C, HLA-DR, CD57, KIR2DL2/DL3, and KIR2DL1 were analyzed in $CD56^+CD3^-$ cells in PBMC samples by flow cytometry. To evaluate the dependence of proportions of different NK cell subsets on IgG titers, cluster analysis was first performed on all the obtained data, resulting in the identification of four main clusters. The identified clusters demonstrated a dependence on the levels of hCMV antibodies, according to which clusters corresponding to seronegative and low-positive were grouped. The results confirmed that hCMV infection leads to an expansion of NK cell populations expressing the NKG2C marker, which correlates with higher levels of IgG response to hCMV. Besides, we identified increased $HLA\text{-}DR^+$ and decreased of $KIR2DL1^+$ NK cells proportions in the middle anti-CMV-IgG level group compared to samples obtained from seronegative and low-positive donors. Moreover, the statistically significant negative correlation was found between $KIR2DL1^+$NK cell percentage and anti-CMV IgG antibody titer, while the positive correlation between $HLA\text{-}DR^+$NK cell proportion and the IgG level was noticed only without the cluster corresponded to high level of anti-hCMV IgG. In this cohort, we did not find any association between KIR2DL3 and CD57 expression in NK cells and levels of IgG response to hCMV. This may indicate that different subsets of NK cells may have distinct roles in regulating humoral immunity to hCMV. Overall, the results of the study provide valuable insights into the coordination of NK cell marker expression and IgG response in hCMV infection.

Keywords: *NK cells, hCMV, IgG, NKG2C, HLA-DR, CD57, KIR2DL2/DL2, KIR2DL1, clusterization assay, correlation assay*

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Introduction

NK cells are able to perform an immune response against virus-infected and tumor cells without prior sensitization [9]. Many activating and inhibitory receptors are involved in the recognition of pathogens and altered cells [7]. The pool of NK cells is characterized by significant phenotypic and functional diversity. However, the phenotype of NK cells changes during differentiation and activation. This process is regulated by the interaction of NK cell receptors with their ligands. Phenotypic and functional diversity is formed not only by the cellular and cytokine environment, but also by the pathogens

that the organism encounters during life. Human cytomegalovirus infection (hCMV) occupies a special place among such pathogens.

hCMV is a ubiquitous herpesvirus that infects approximately 60% of adults worldwide. While usually asymptomatic in healthy individuals, hCMV can cause severe disease and even death in immunocompromised patients, such as transplant recipients or those with HIV/AIDS [3]. Despite its prevalence and clinical significance, the immune response to hCMV remains poorly understood.

NK cells play an important role in immune defense against human cytomegalovirus infection (hCMV). NK cells express various activating and inhibitory receptors, which allow them to recognize and eliminate virus-infected cells while sparing healthy cells. Recently, a special subpopulation of "adaptive" NKG2C$^+$NK cells was identified that reacts to hCMV infection and possesses some properties of immunological memory [2]. The term "immunological memory" implies an enhanced or in some way reprogrammed, prolonged and antigen-specific response. Adaptive NKG2C$^+$NK cells are highly differentiated; they express carbohydrate antigen CD57 and the KIR family receptors [5]. The HLA-DR as an essential activating receptor on the surface of NK cells [1] may also characterize the response of NK cells to hCMV. In addition, hCMV-specific antibodies, particularly of the IgG isotype, are crucial for controlling hCMV replication and preventing disease progression [10].

However, the exact interplay between NK cell activation and the humoral immune response in hCMV infection is not well understood. Understanding this connection could be crucial for developing more effective therapies against hCMV. Therefore, in this study, we investigate the potential coordination of NK cell markers expression and IgG response in hCMV infection. By exploring the connection between these two arms of the immune system, we hope to provide new insights into the immune response to hCMV.

Materials and methods

1. Samples

Peripheral blood mononuclear cells (PBMC) and plasma samples of volunteer healthy adults of different genders with median age 31 years were collected. Oral informed consent to participate in the study was received from each donor. The participant cohort included 33 subjects, the main characteristics of which are listed in Table 1.

TABLE 1. CHARACTERISTICS OF HEALTHY VOLUNTEERS

Donor	Gender	Age	IgG to hCMV
1	female	53	13.448
2	female	24	0
3	female	38	3.388
4	female	61	3.558
5	female	54	1.958
6	male	57	3.498
7	female	22	0.792
8	male	42	2.448
9	female	69	3.832
10	female	24	4.894
11	male	45	1.364
12	female	23	1.358
13	female	26	8.954
14	male	28	0
15	female	18	9.616
16	female	49	12.732
17	male	65	0
18	male	23	0
19	female	30	1.272
20	female	25	8.763
21	female	27	0.398
22	male	59	7.942
23	female	27	0
24	female	31	12.656
25	male	27	8.672
26	female	30	0.359
27	female	48	3.515
28	female	53	9.985
29	male	27	2.82
30	female	29	8.788
31	female	41	5.731
32	female	39	7.582
33	female	59	9.935

2. ELISA

The hCMV-specific IgG levels in plasma samples of the healthy volunteers were measured using hCMV IgG Fluorescent Immunoassay kit (Vector-Best, Novosibirsk, Russia).

3. Phenotype analysis

PBMC samples were stained with the following fluorescent-labeled antibodies: CD3-PerCP (clone HIT3a, Sony Biotechnology San Jose, CA, USA), CD56-APC-Vio770 (clone REA196, Miltenyi Biotec, Bergisch Gladbach, Germany), NKG2C-FITC (clone REA205, Miltenyi Biotec, Bergisch Gladbach, Germany), HLA-DR-PE-Vio770 (clone REA332, Miltenyi Biotec, Bergisch Gladbach, Germany), KIR2DL2/L3-APC (clone DX27, Sony Biotechnology, San Jose, CA, USA), CD57-Vio-Blue (clone TB03, Miltenyi Biotec, Bergisch Gladbach, Germany), KIR2DL1-PE (clone HP-MA4, Sony Biotechnology, San Jose, CA, USA). Samples were analyzed using a MACSQuant 10 flow cytometer (Miltenyi Biotec, Germany) equipped with lasers $\lambda = 405$ nm, $\lambda = 488$ nm, $\lambda = 635$ nm.

4. Statistical analysis

The data was analyzed using FlowJo, GraphPad Prism X 10.0.7r2, and R language. The clusterization analysis was performed via construction of a heatmap using the library (pheatmap), which constructs a heatmap based on the numeric values in the sample matrix. The "row Z-score" scaling method was used to scale each row to have a mean of 0 and a standard deviation of 1. The analysis of percentages was carried out with a nonparametric Kruskal-Wallis test. Correlation analysis was done using Spearman correlation for nonparametric samples. The value of $p < 0.05$ was considered statistically significant.

Results and discussion

1. Clusterization of samples with following phenotypic analysis

Surface expression levels of NKG2C, HLA-DR, CD57, and KIR2DL2/DL3 in NK cells were analyzed in PBMC samples obtained from 33 volunteer healthy adults by flow cytometry. NK cells were gated as $CD56^+CD3^-$ cells. The hCMV serological status was determined by ELISA kit to hCMV-specific IgG level. Pre-log normalized data on anti-hCMV IgG antibody titers together with the percentages of the NKG2C, HLA-DR, CD57, and KIR2DL2/DL3 were used for clustered heatmap analysis with hierarchical relationships between samples in order to divide the donor into groups (Figure 1A). The four main clusters were identified (Figure 1A) and anti-hCMV IgG antibody titer to hCMV among clusters was analyzed. The highest anti-hCMV IgG antibody titers were found in cluster 4, which significantly differed from the cluster 1 and cluster 2 IgG levels (Figure 1B). The cluster 3 also showed higher anti-hCMV IgG levels compared to the cluster 1. The cluster 1 corresponded to the sero-negative status of donors, and the cluster 2 represented the low-seropositive hCMV status. Taking into account that no differences were observed between the clusters 1 and 2 (Figure 1B), for further analysis of relationships of the IgG levels and NK cell subset proportions the clusters 1 and 2 were combined.

The highest percentage of $NKG2C^+$NK cells was observed in cluster 4 (Figure 1C), which has been noticed by different research groups previously [4, 6, 8]. Another important thing in antiviral response is the activation state of the cells. The HLA-DR is an essential activation marker of NK cells [1]. The HLA-DR expression was increased in samples of cluster 3 compared to the cluster 1 and 2, while there were no differences between cluster 4 and other clusters (Figure 1D). We revealed that the percentage of $HLA\text{-}DR^+$NK cells was increased in cluster 3 with middle anti-hCMV IgG antibody titers compared to undetectable and low anti-hCMV IgG antibody titers, which indicated that the NK cells were activated in cluster 3. At the same time, in cluster 4 containing samples with high anti-hCMV IgG antibody titers the percentage of $HLA\text{-}DR^+$NK cells did not differ significantly compared to samples from clusters 1 and 2 (Figure 1D). These findings may indicate that higher adaptive B cells immune response is associated with lower NK cell activation level and vice versa the high NK cells response indicates lower adaptive B cells response. We did not find significant differences in the percentages of both $KIR2DL2/DL3^+$ and $CD57^+$NK cells between all studied groups (Figure 1E, F). Additionally, we decided to analyze the KIR2DL1 expression in NK cells in part of the donors. The level of $KIR2DL1^+$NK cells showed the tendency to decrease in cluster 3 compared to the group that united clusters with undetectable and low anti-hCMV IgG antibody levels (Figure 1G).

2. Correlations between NK cell markers and anti-hCMV IgG antibody titers

The Spearman correlation assay of $NKG2C^+$, $HLA\text{-}DR^+$, $CD57^+$, $KIR2DL2/DL3^+$, and $KIR2DL1^+$NK cell percentages vs anti-hCMV IgG antibody titers

was performed. Positive correlation was identified between NKG2C$^+$ cells and hCMV-specific IgG levels (Figure 2A). It was shown earlier that the NKG2C expression is mostly associated with CD57 expression in hCMV infection [4]. However, we have not observed any dependence of CD57 expression on anti-hCMV IgG antibody titers (Figure 2C). By contrast, a negative correlation was observed between KIR2DL1 expression and anti-hCMV IgG antibody titer (Figure 2B). No correlations were found for KIR2DL2/DL3 as well as HLA-DR to IgG to CMV (Figure 2D, E). However, further analysis of HLA-DR expression on the samples from clusters 1, 2 and 3 revealed the positive correlation between HLA-DR

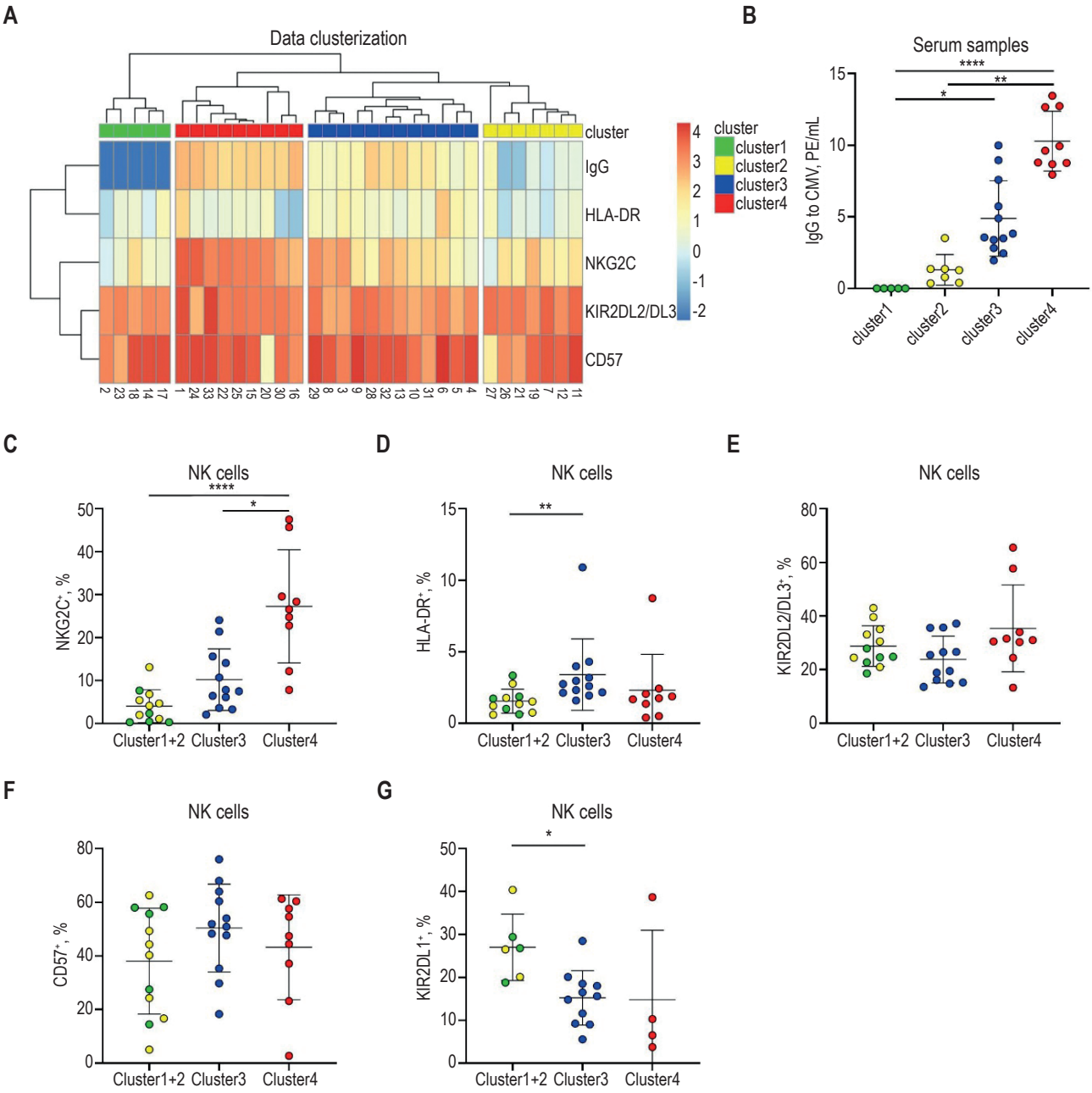

Figure 1. Clustering analysis of the NK cells phenotype in hCMV infection

Note. (A) Hierarchical clustering of log-normalized anti-hCMV IgG antibody titers and percentage of HLA-DR, NKG2C, CD57 and KIR2DL2/DL3 NK cells for 33 samples using heatmap reveals 4 discrete sample-level clusters. (B) anti-hCMV IgG antibody titers to hCMV in PE/ml among 4 clusters. (C) NKG2C expression on NK cells among 4 clusters. (D) HLA-DR expression on NK cells among 4 clusters. (E) KIR2DL2/DL3 expression on NK cells among 4 clusters. (F) CD57 expression on NK cells among 4 clusters. (G) KIR2DL1 expression on NK cells among 4 clusters. Data are presented as the mean (± SD). Kruskal–Wallis statistical tests for nonparametric samples were used. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

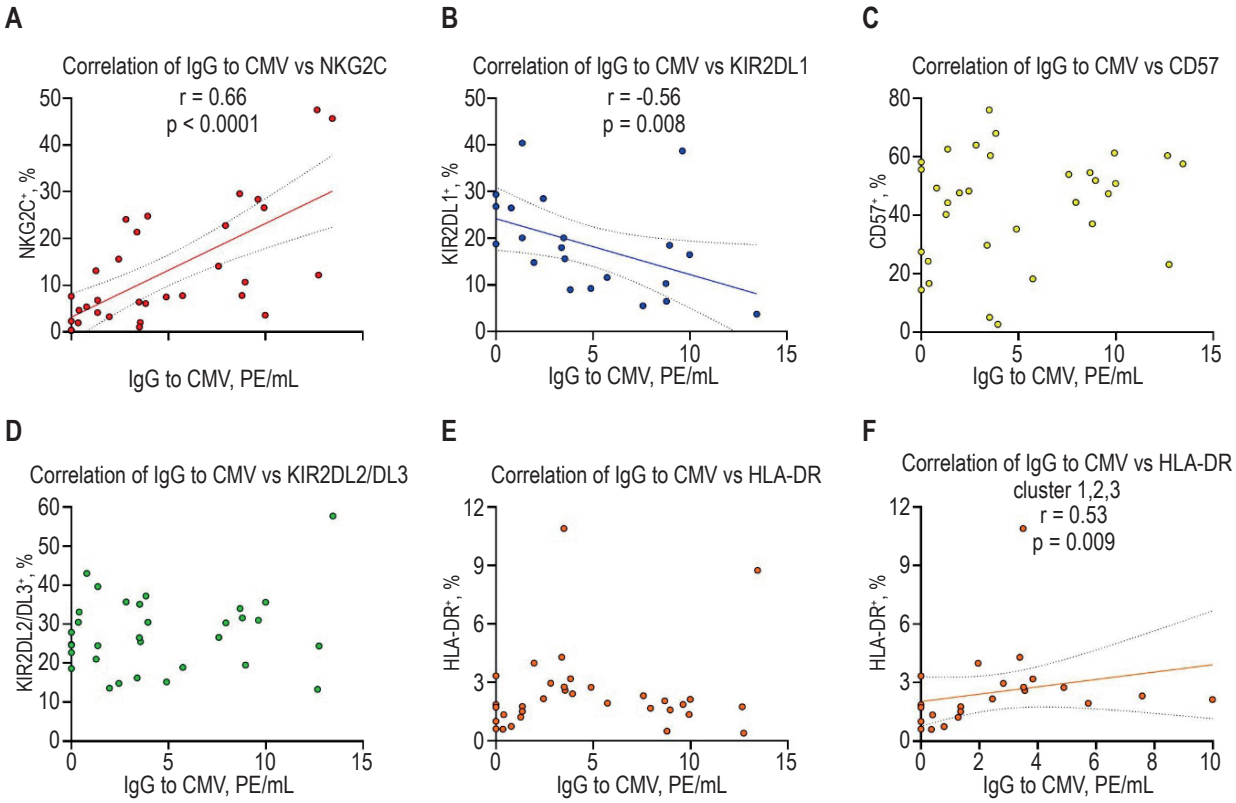

Figure 2. Correlation assay of NK cells in hCMV infection

Note. (A) Spearman correlation between the percentage of NKG2C and IgG to CMV in PE/mL. (B) Spearman correlation between the percentage of KIR2DL1 and IgG to CMV in PE/mL. (C) Spearman correlation between the percentage of CD57 and IgG to CMV in PE/mL. (D) Spearman correlation between the percentage of KIR2DL/2DL3 and IgG to CMV in PE/mL. (E) Spearman correlation between the percentage of HLA-DR and IgG to CMV in PE/mL. (F) Spearman correlation between the percentage of HLA-DR and IgG to CMV in PE/mL among clusters 1, 2, and 3.

and anti-hCMV IgG antibody titers (Figure 2F). What may possibly indicate that the high adaptive B cells immune response is associated with lower degree of NK cells activation, while the high NK cells response corresponds to the indicated lower adaptive B cells response.

Conclusion

Further research is needed to fully understand the role of NK cells in the complex immune response to hCMV infection. Taken together, our results indicate the coordination of NKG2C, HLA-DR, and KIR2DL1 expression in hCMV infection.

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