АНАЛИЗ КЛОНАЛЬНЫХ ПОПУЛЯЦИЙ NK-КЛЕТОК НА ОСНОВЕ ДАННЫХ SINGLE-CELL ТРАНСКРИПТОМИКИ

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Резюме. Естественные киллеры (NK-клетки) являются ключевым компонентом противовирусного иммунитета, демонстрируя исключительную адаптивность при таких инфекциях, как цитомегаловирус человека (hCMV) и SARS-CoV-2. Современные методы single-cell транскриптомики выявили существование клональных популяций NK-клеток с уникальными функциональными профилями, что стирает традиционные границы между врожденным и адаптивным иммунитетом. Однако функциональная гетерогенность и иммунологическая значимость этих клонов остаются недостаточно изученными. Данное исследование направлено на анализ клональной гетерогенности NK-клеток с использованием опубликованных данных scRNA-seq от hCMV-серопозитивных, серонегативных доноров и пациентов с COVID-19, с акцентом на кластер-специфические паттерны экспрессии генов. В работе применялся вычислительный pipeline на основе Seurat для интеграции и кластеризации данных высокого разрешения от hCMV-серопозитивных (n = 5) и серонегативных (n = 2) доноров, а также пациентов с COVID-19 (n = 2). Данные анализировали в Seurat 5 (R) после контроля качества, нормализации (SCTransform), интеграции (коррекция batch-эффектов) и кластеризации (UMAP). Проводили анализ дифференциальной экспрессии генов (критерии: тест Вилкоксона, log2FC > 0,25, p-adj < 0,05) с последующей аннотацией. У hCMV-серопозитивных доноров выявлено 12 транскрипционно различных кластеров NK-клеток с *KLRC2* (NKG2C)-зависимой организацией, где отдельные клоны демонстрировали либо повышенный цитотоксический потенциал (активация *GZMB/GZMA*), либо уникальные профили ингибиторных рецепторов (вариабельная экспрессия KIR). В серонегативной группе обнаружена более простая клональная структура (9 кластеров) с уменьшенным разнообразием KIR, но сохранением характерных эффекторных генетических сигнатур. У пациентов с COVID-19 выявлены различные клональные паттерны: у одного наблюдалась сниженная вариабельность *KLRC2* при выраженной экспрессии *KLRC1* (NKG2A), тогда как у другого отмечалась гетерогенность KIR без вариаций *KLRC2*. Наше исследование демонстрирует существование различных

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клональных популяций NK-клеток при hCMV-инфекции и COVID-19, характеризующихся дивергентной экспрессией активирующих и ингибиторных рецепторов. Полученные данные раскрывают инфекционно-специфическую динамику клональных популяций NK-клеток, подчеркивая их адаптивный потенциал через дифференциальную экспрессию рецепторов в противовирусных ответах.

Ключевые слова: NK-клетки, scRNAseq, hCMV, COVID-19, NKG2, KIR

ANALYSIS OF CLONAL NK CELL POPULATIONS USING SINGLE-CELL TRANSCRIPTOMICS DATA

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Abstract. Natural killer (NK) cells represent a critical component of antiviral immunity, demonstrating remarkable adaptability during infections such as human cytomegalovirus (hCMV) and SARS-CoV-2. Recent advances in single-cell transcriptomics have revealed the clonally expanding NK cell populations with distinct functional profiles, blurring the traditional boundaries between innate and adaptive immunity. However, the functional heterogeneity and immunological significance of these clones remain incompletely understood. Our aim was to dissect clonal NK cell heterogeneity using published scRNA-seq datasets from hCMV-seropositive, seronegative, and COVID-19 patients, focusing on cluster-specific gene expression patterns. Our computational pipeline employed Seurat-based integration and high-resolution clustering of datasets from hCMV-seropositive (n = 5) and seronegative (n = 2) donors, along with COVID-19 patients (n = 2). We analyzed datasets using Seurat 5 in R. Quality-controlled data were normalized (SCTransform), integrated (batch-corrected), and clustered (UMAP). Differential gene expression (Wilcoxon test, log2FC > 0.25, p-adj < 0.05) and annotation were performed. In hCMV-seropositive individuals, we identified 12 transcriptionally distinct NK cell clusters exhibiting *KLRC2* (NKG2C)-dependent organization, with specific clones showing either enhanced cytotoxic potential (marked by *GZMB/GZMA* upregulation), or unique inhibitory receptor profiles (variable KIR expression patterns). The hCMV-seronegative cohort displayed a simpler clonal structure with 9 clusters showing reduced KIR diversity but maintained distinct effector gene signatures. Analysis of COVID-19 patients revealed divergent clonal patterns: one patient showed reduced *KLRC2* variability with prominent *KLRC1* (NKG2A) expression, while another exhibited KIR heterogeneity without *KLRC2* variation. Our analysis reveals distinct clonal NK cell populations in hCMV and COVID-19 contexts, characterized by divergent expression of activating and inhibitory receptors. These findings demonstrate infection-specific dynamics of clonal NK cell populations, highlighting their adaptive potential through differential receptor expression in antiviral responses.

Keywords: NK cells, scRNAseq, hCMV, COVID-19, NKG2, KIR

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Introduction

Natural killer (NK) cells play a critical role in antiviral immune responses, including those against human cytomegalovirus (hCMV) [3, 7] infection and severe acute respiratory syndrome (COVID-19) [5, 8, 9, 10]. In recent years, single-cell RNA sequencing (scRNA-seq) has revealed the heterogeneity of NK cells, including the existence of clonally expanded populations with unique transcriptional profiles [6].

During CMV infection in both mice and humans, memory NK cell populations emerge through clonal expansion. In mice, the clonal nature of memory NK cells was confirmed using retroviral barcoding with fluorescent labeling to track individual Ly49H$^+$NK cells. After transfer into immunodeficient recipients followed by mCMV infection, these labeled Ly49H$^+$NK cells underwent extensive proliferation, with some clones exceeding 10,000 cells [2].

In humans, clonal expansion of adaptive NKG2C$^+$NK cells were indicated by a distinct pattern of activating KIR receptors associated with self-antigen recognition [1]. Further insights were obtained using mitochondrial DNA mutation tracking, a method previously employed for clonal lineage analysis [4]. Notably, T. R ckert et al. demonstrated that many large clusters identified by scATAC-Seq represent clonal NK cells [6]. These findings highlight the ability of NK cells to form clonal populations, aligning them more closely with adaptive immune cells and underscoring their role in long-term immune defense.

However, the detailed characterization of these clones, including their functional properties and role in immune responses, remains poorly understood. In this study, we analyzed clonal NK cell populations using published scRNA-seq datasets. We identified and compared NK cell clusters in hCMV-seropositive and seronegative donors, as well as in COVID-19 patients. Key differentially expressed genes associated with NK cell function were also determined.

Materials and methods

Data sources

The analysis was performed using publicly available single-cell RNA sequencing datasets obtained from the Gene Expression Omnibus database under accession numbers GSE197037 and GSE184329.

Initial data processing

Initial data processing involved standard quality control measures to filter out low-quality cells, including those with abnormally high mitochondrial gene content or an insufficient number of detected genes. The data were then normalized using the SCTransform method implemented in Seurat 5. To account for technical variation between different samples and donors, we performed dataset integration function in Seurat 5, which effectively corrects for batch effects while preserving biologically relevant variation.

Computational analysis

For dimensionality reduction and visualization, we applied the UMAP algorithm with clustering resolutions set to 1.5 for hCMV datasets and 1.0 for COVID-19 datasets to optimally identify distinct cell populations. Differential gene expression analysis was conducted using the Wilcoxon rank-sum test with Benjamini-Hochberg multiple testing correction, applying thresholds of absolute log2 fold change greater than 0.25 and adjusted p-values below 0.05 for statistical significance. Identified genes were subsequently annotated using Gene Ontology.

Software implementation

All analyses were conducted using R version 4.4.0, primarily utilizing the Seurat 5 package for single-cell data processing and analysis. Data visualization was achieved through ggplot2, while data manipulation and organization were performed using dplyr.

Results and discussion

Analysis of clonal NK cells in hCMV-seropositive and seronegative donors

The initial phase of our investigation focused on characterizing clonal NK cell populations using publicly available single-cell transcriptomics (scRNA-seq) data. We processed with the data from T. R ckert et al. [6] that included NK cell profiles from five hCMV-seropositive and two hCMV-seronegative donors. The original study had examined balanced mixtures of NKG2C$^+$ and NKG2C$^-$NK cell subpopulations (CD3$^-$CD14$^-$CD19$^-$CD7$^+$) at a 1:1 ratio from each donor.

While following the general analytical approach of the original study, we introduced modifications including enhanced UMAP resolution parameters to improve detection of clonal structures. This refined analysis identified 12 distinct NK cell clusters in hCMV-seropositive donors (Figure 1A, B), which were consistently present across all individuals but showed variable proportional representation. The hCMV-seronegative cohort exhibited 9 clusters with similar inter-individual variability (Figure 1C, D).

Focusing on clonal NK cell characterization, we particularly examined functionally relevant genes. In seropositive donors, UMAP separation was primarily driven by *KLRC2* expression (encoding NKG2C) (Figure 1E). However, the 12 clusters demonstrated additional transcriptional differences. For instance, while both Cluster 2 and Cluster 0 showed low *KLRC2* expression, Cluster 2 displayed elevated CD38 and CD160 levels, compared to Cluster 0. The upregulation of those genes highlights the higher activation stage of those NK cells. Additionally, Cluster 2 has shown the downregulation of some inhibitory KIRs (KIR2DL3 and KIR3DL1) (Figure 1E). That may suggest that such clusters of NK cells expressing different KIRs receptors may belongs to originally different clonal NK cells.

Among *KLRC2*-high clusters, Cluster 3 exhibited markedly increased expression of cytotoxic effector genes molecules *GZMB* and *GZMA* along with *KLRG1* compared to Cluster 1 (Figure 1E). Thus, NK cells from Cluster 3 are probably more cytotoxically active.

Simmilar gene expression patterns were observed in seronegative donors (Figure 1F). While hCMV-seropositive donors showed *KLRC2*-driven clustering, seronegative donors lacked this pattern and had reduced KIR expression (Figure 1F). However, all clusters retained distinct NK cell-associated gene signatures. (Figure 1F). Nevertheless, each cluster displayed a unique combination of NK cell-associated genes.

COVID-19 patient NK cell clonal analysis

We subsequently analyzed scRNA-seq data from M. Witkowski et al. [11] on NK cell in COVID-19

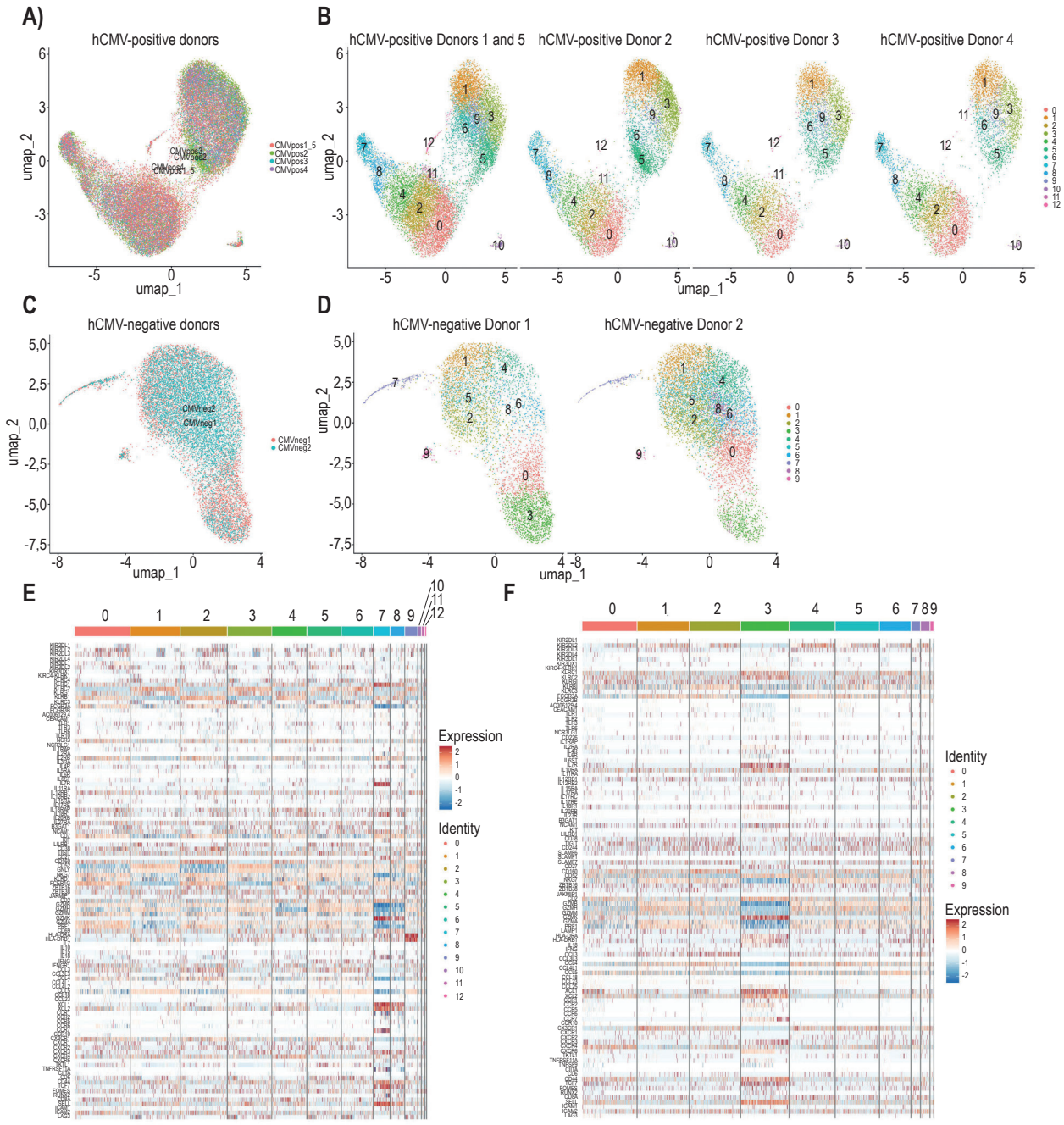

Figure 1. Single-cell RNA sequencing analysis of 5 hCMV-seropositive and 2 hCMV-seronegative donors from [6]

Note. A, UMAP projection of NK cells from 5 hCMV-seropositive donors showing donor-specific groupings. B, UMAP projection of NK cells from 2 hCMV-seronegative donors displaying clusters for each donor in the integrated dataset. C, UMAP projection of NK cells from 2 hCMV-seronegative donors illustrating donor groupings. D, UMAP projection of NK cells from 5 hCMV-seropositive donors showing clusters for each donor in the integrated dataset. Heatmaps depict normalized expression values of NK cell-associated genes that were differentially expressed in at least one cluster for (E) 5 hCMV-seropositive donors and (F) 2 hCMV-seronegative donors.

patients, selecting two patients with the highest cell yields. After removing cells with elevated mitochondrial gene expression and erythrocyte contaminants, high-resolution UMAP projection revealed 13 clusters in the data from first donor and 11 from the second (Figure 2A, B).

We performed comparable differential expression analysis of NK cell-associated genes across these identified clusters (Figure 2C, D). For Patient 1, there was less variability in *KLRC2* gene expression compared to hCMV-seropositive donors, while the expression of *KLRC1* (encoding the inhibitory

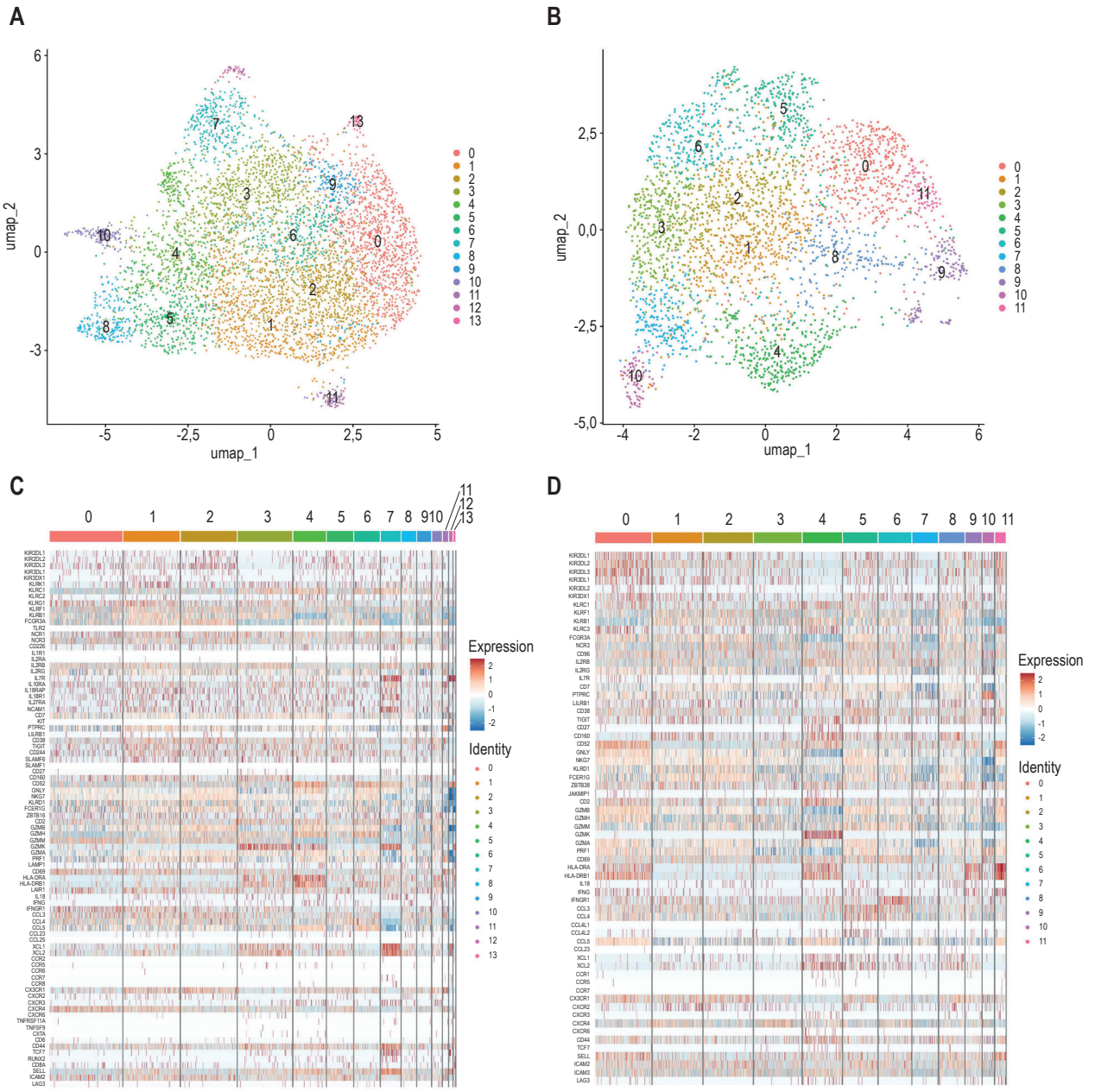

Figure 2. Single-cell RNA sequencing analysis of NK cells from two patients from [11]

Note. UMAP projections showing NK cell clusters for (A) patient1 and (B) patient 2. Heatmaps display normalized expression values of NK cell-associated genes differentially expressed in at least one cluster for (C) patient 1 and (D) patient 2.

NKG2A receptor) was more pronounced (Figure 2C). Additionally, fewer KIRs were variable compared to hCMV-seropositive donors (Figure 2C). Moreover, Patient 2 did not show any variability in *KLRC1* expression, while exhibiting high expression of KIRs, which varied among the clusters (Figure 2D).

Conclusion

Thus, our study revealed the infection-specific dynamics of clonal NK cell populations, highlighting their adaptive potential through differential receptor expression in antiviral responses.

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