Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Abnormality in the NK-cell population is prolonged in severe COVID-19 patients

Galam Leem, MD, PhD, Shinhye Cheon, MD, PhD, Hoyoung Lee, PhD, Seong Jin Choi, MD, PhD, Seong Jeong, BS, Eui-Soon Kim, MD, Hye Won Jeong, MD, PhD, Hyeongseok Jeong, MD, MS, Su-Hyung Park, PhD, Yeon-Sook Kim, MD, PhD, and Eui-Cheol Shin, MD, PhD

Seoul, Daejeon, and Cheongju, Korea

Background: Our understanding of adaptive immune responses in patients with coronavirus disease 2019 (COVID-19) is rapidly evolving, but information on the innate immune responses by natural killer (NK) cells is still insufficient.

Objective: We aimed to examine the phenotypic and functional status of NK cells and their changes during the course of mild and severe COVID-19.

Methods: We performed RNA sequencing and flow cytometric analysis of NK cells from patients with mild and severe COVID-19.

Disclosed potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Data and materials availability: RNA sequencing data have been uploaded in the Gene Expression Omnibus (GEO) database under GEO accession number GSE165461. All other data that support the findings of this study and materials are available on request.

Received for publication February 21, 2021; revised June 27, 2021; accepted for publication July 21, 2021.

Available online July 31, 2021.

Corresponding author: Yeon-Sook Kim, MD, PhD, Division of Infectious Diseases, Department of Internal Medicine, Chungnam National University School of Medicine, 282 Munhwa-ro, Jung-gu, Daejeon 35015, Korea. E-mail: alice@cmuh.co.kr; Or: Eui-Cheol Shin, MD, PhD, Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology, 291 Daehak-ro, Yuseong-gu, Daejeon 34141, Korea. E-mail: ecsinh@kaist.ac.kr.

The CrossMark symbol notifies online readers when updates have been made to the article such as errata or minor corrections.

© 2021 American Academy of Allergy, Asthma & Immunology
https://doi.org/10.1016/j.jaci.2021.07.022
A worldwide pandemic of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has continued uncasingly. Since a cluster of atypical pneumonia cases of unknown origin, now identified as being caused by SARS-CoV-2, were reported in Wuhan city, Hubei province, China, in December 2019, more than 90 million confirmed cases and more than 2 million deaths have been reported globally as of January 19, 2021. Most patients with coronavirus disease 2019 (COVID-19), which is caused by SARS-CoV-2 infection, recover spontaneously, complaining of only mild symptoms, but some patients, especially elderly patients, develop severe manifestations, including acute respiratory distress syndrome, multiorgan failure, and death. Our understanding of the immune responses against SARS-CoV-2 has deepened significantly, but the responses of NK cells and the innate lymphocytes are still unclear.

Natural killer (NK) cells are a major subset of innate lymphocytes and play an important role in early protection against viruses and the regulation of the cellular and humoral adaptive immune responses. NK cells are conventionally subdivided into 2 subsets on the basis of relative surface expression of CD56 and CD16: the CD56\textsuperscript{dim}CD16\textsuperscript{pos} NK-cell subset, which is predominant in peripheral blood and considered to be the cytolytic NK-cell subset, and the CD56\textsuperscript{bright}CD16\textsuperscript{neg} NK-cell subset, which is predominant in the secondary lymphoid tissues and considered to be the cytokine-producing NK-cell subset. Recently, several studies have reported the presence of the unconventional CD56\textsuperscript{dim}CD16\textsuperscript{neg} (uCD56\textsuperscript{dim}) NK-cell population in the peripheral blood of healthy donors and showed that the population can be expanded in several clinical conditions. However, little is currently known about the role of this uCD56\textsuperscript{dim} NK-cell population in acute viral infection.

In patients with COVID-19, the absolute number of NK cells is reduced in line with general lymphopenia. The Karolinska COVID-19 Study Group reported that NK cells in patients with COVID-19 exhibit activated phenotypes (higher expression of Ki-67, HLA-DR, and CD69 than in healthy controls) and are enriched with “activation/proliferation signatures” and an “effector function gene signature,” whereas others have reported that NK cells present exhausted phenotypes (higher expression of T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) and programmed cell death protein 1 (PD-1) than in healthy donors) and are functionally impaired.

In the present study, we examined the phenotypic and functional status of NK cells and their changes during the course of mild and severe COVID-19. We performed flow cytometric analysis and RNA sequencing (RNA-seq) using cryopreserved PBMCs from patients with mild and severe COVID-19 at several serial time points. We found that a unique subset of NK cells, uCD56\textsuperscript{dim} NK cells, is characterizedly expanded in patients with COVID-19. In addition, we demonstrate that the cytotoxicity of NK cells is decreased in patients with COVID-19. During the course of COVID-19, uCD56\textsuperscript{dim} NK cells disappear rapidly in mild patients but slowly in severe patients, and the cytotoxicity of NK cells recovers accordingly.

**METHODS**

**Study design**

We prospectively enrolled patients diagnosed with COVID-19 from Changnam National University Hospital, Daejeon, Korea. Fresh blood and plasma samples were obtained at several time points during hospitalization. Table E1 in this article’s Online Repository at www.jacionline.org presents the clinical characteristics of the enrolled patients and healthy donors. Table E2 in this article’s Online Repository at www.jacionline.org presents the sampling time points and the assessment assays in which the samples were used. The time was counted from the day of diagnosis (day 1). The patients with COVID-19 were diagnosed an average 9.9 days from symptom onset. Table E3 in this article’s Online Repository at www.jacionline.org presents the initial laboratory test results of the enrolled patients with COVID-19. SARS-CoV-2 RNA was detected in patients’ nasopharyngeal swab and sputum specimens by real-time PCR using the PowerChek 2019-nCoV Real-time PCR Kit (Kogenebiotech, Seoul, Korea). Hospitalized patients diagnosed with acute influenza A virus infection by a rapid antigen test of a nasopharyngeal swab were also enrolled from Chungbuk National University Hospital from December 2015 to April 2016 before the emergence of COVID-19. We collected samples from healthy donors and patients with influenza before the emergence of COVID-19, so we reasonably assumed that they were free from SARS-CoV-2 infection.

All patients provided written informed consent. This study was conducted in accordance with the Declaration of Helsinki (1996) and approved by the institutional review boards of all participating institutions.

All resources including antibodies and agents used in this study are presented in Table E4 in this article’s Online Repository at www.jacionline.org.

**Isolation of PBMCs and NK cells**

Using whole blood samples obtained from patients with COVID-19, patients with influenza A, and healthy donors, we isolated PBMCs by Ficoll-Paque density gradient centrifugation using lymphocyte separation medium (Corning, New York, NY). PBMCs were cryopreserved until use.
FIG 1. Transcriptome analysis of NK cells from healthy donors and patients with COVID-19. RNA-seq of sorted CD3−CD56+ NK cells from cryopreserved/thawed PBMCs obtained from 8 healthy donors, 13 patients with mild COVID-19, and 8 patients with severe COVID-19 at the earliest time point for each. A and B, Volcano plot (Fig 1, A) and heatmap (Fig 1, B) showing upregulated or downregulated genes between healthy donors and patients with COVID-19: 217 genes were upregulated (cluster 1) and 208 genes were downregulated (cluster 2) in patients with COVID-19. Representative genes are annotated. C, KEGG pathway analysis of cluster 1. The top 5 pathways are presented with the odds ratio (blue bar) and −log(P value) (orange bar). D, Bar graphs showing the enrichment P values of the top 10 GO biological processes for cluster 1. GO IDs are annotated. E, Bar graphs showing the enrichment P values of the top 10 cytokine-responsive gene signatures from the L1000 LINCS database for cluster 1. Gene signatures are presented in the order of the cytokines (bold) and the name of the cytokine-treated cell lines. F, Protein-protein interaction network analysis of the top 10 cluster 1–related transcription factors. The size of the circle represents the odds ratio, and the fill color represents the value of −log(P value). GO, Gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; TF, transcription factor.
Healthy Influenza Mild COVID-19 Severe COVID-19

1.39

1 : CD56$^{\text{bright}}$ CD16$^{\text{neg}}$ NK cells (cCD56$^{\text{bright}}$)
2 : CD56$^{\text{dim}}$ CD16$^{\text{pos}}$ NK cells (cCD56$^{\text{dim}}$)
3 : CD56$^{\text{dim}}$ CD16$^{\text{neg}}$ NK cells (uCD56$^{\text{dim}}$)

FIG 2. Identification of NK-cell subsets and phenotypes of each subset using flow cytometry. Data from samples obtained on the day of diagnosis (day 1) are presented for patients with COVID-19 (A-D). A, Representative flow cytometry plots showing the gating strategy for CD3–CD56$^{+}$ NK cells on the left. Right bar plots show cumulative data regarding the frequency of NK cells among CD14–CD19– lymphocytes in healthy donors (green bar, n = 13), patients with influenza (blue bar, n = 7), patients with mild COVID-19 (orange bar, n = 17), and patients with severe COVID-19 (red bar, n = 8). B, Flow cytometry plots of NK cells from a representative healthy donor, patients with influenza, patients with mild COVID-19, and patients with severe COVID-19 with identification of each NK-cell subset: subset 1 represents cCD56$^{\text{bright}}$ NK cells, subset 2 cCD56$^{\text{dim}}$ NK cells, and subset 3 uCD56$^{\text{dim}}$ NK cells. The frequencies of each subset among NK cells are presented. C, Bar plots showing cumulative data regarding the relative frequencies of each subset among NK cells in healthy donors (green bar), patients with influenza (blue bar), patients with mild COVID-19 (orange bar), and patients with severe COVID-19 (red bar). D, Pie charts showing the average frequency of each NK-cell subset by disease group: green slice for cCD56$^{\text{bright}}$, blue slice for cCD56$^{\text{dim}}$, and red slice for uCD56$^{\text{dim}}$ NK cells. E, Expansion of the uCD56$^{\text{dim}}$ NK-cell population during the early acute phase of COVID-19. Flow cytometry plots for 2 patients (Pt 4 and Pt 6) on the day of symptom onset, 2 days after symptom onset, and 4 days after symptom onset are presented. FSC-H, Forward scatter-height; FSC-A, Forward scatter-area; SSC-A, Side scatter-area. *P < .05. **P < .01. ***P < .001. ****P < .0001.
FIG 3. Intracellular cytokine staining to evaluate the proliferating and cytolytic capacities of each NK-cell subset and assessment of NK-cell cytotoxicity the first week after diagnosis. A, Bar plots showing the cumulative data regarding the expression of NK-cell receptors in each NK-cell subset: cCD56bright (green bar), cCD56dim (blue bar), and uCD56dim NK cells (red bar). Data from 9 healthy donors, 7 patients with influenza, 6 patients with mild COVID-19, and 7 patients with severe COVID-19 are presented. The expression of NKp30, NKp44, NKp46, KIRs (KIR2D and KIR3DL1/2), TIGIT, NKG2A, and NKG2C is presented as the relative frequency (%). B, Flow cytometry plots showing the expression of CD16 and HLA-DR in cCD56dim NK cells. C, Flow cytometry plots showing the expression of Ki-67, Perforin, and GranzymeB in cCD56bright, cCD56dim, and uCD56dim NK cells. D, Graph showing the specific lysis (%) in each NK-cell subset at different E:T ratios (0.25, 1.25, 2.5, 5, 10, and 20). E, Graph showing the specific lysis (%) in severe COVID-19 patients at an E:T ratio of 10:1. The expression of granzyme B and perforin is presented as the gMFI. The frequency of uCD56dim NK cells is presented as the x-axis, and the specific lysis (%) is presented as the y-axis. The R² value is 0.5719, and the P value is 0.0028.
After thawing cryopreserved PBMCs, the cell viability was 86.88% ± 7.35% (healthy donors), 69.21% ± 13.77% (patients with influenza), 79.75% ± 12.11% (patients with mild COVID-19), and 75.22% ± 10.19% (patients with severe COVID-19) (see Table E5 in this article’s Online Repository at www.jacionline.org). Thawed PBMCs were directly analyzed without in vitro incubation.

NK cells were isolated 2 ways. For RNA-seq, we stained PBMCs with antibodies against 7-AAD, CD3, and CD56, and sorted NK cells on the basis of surface expression of CD3 and CD56 (CD3–CD56+) using an ARIA II cell sorter. The gating strategies for NK-cell sorting are presented in Fig E1 in this article’s Online Repository at www.jacionline.org. For the NK-cell cytotoxicity assay, to avoid affecting NK cells as much as possible, we negatively isolated NK cells using the NK-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). We stained other cells except NK cells in the PBMCs according to the manufacturer’s instructions and obtained NK cells by removing magnetically labeled cells using a magnetic-activated cell sorter (Miltenyi Biotec).

RNA-seq

RNA-seq was performed using sorted CD3–CD56+ NK cells from cryopreserved/thawed PBMCs obtained from patients with COVID-19 and healthy donors as described above. The sorted CD3–CD56+ NK cells were immediately dissolved in TRIzol LS reagent (Invitrogen, Waltham, Mass), and RNA extraction was performed following the manufacturer’s instructions. RNA quality was assessed by a bioanalyzer system (Agilent 2100, Agilent Scientific Instrument, Santa Clara, Calif), and an RNA library was constructed using the QuantSeq 3’ mRNA-Seq Library Prep Kit (Lexogen, Vienna, Austria). Sequencing was performed using a NextSeq 550; the sequencing reads were aligned to the human reference genome GRCh38 using STAR (v 2.7.2) and normalized for effective library size. We analyzed differentially expressed genes using the DESeq2 algorithm21 and defined them on the basis of a P value of less than .05 and log2 fold change of more than 1.

Gene set enrichment analysis was performed using web-based software Enrichr.22,23 The access date for the Kyoto Encyclopedia of Genes and Genomes and gene ontology analyses was January 4, 2021.

The RNA sequencing data have been uploaded to the Gene Expression Omnibus database under Gene Expression Omnibus accession number GSE165461.

Flow cytometry and intracellular cytokine staining

For surface protein staining, cryopreserved PBMCs were thawed and stained first using the LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen) for 5 minutes at room temperature in the dark. Without washing, the LIVE/DEAD-stained cells were stained with multiple fluorescein-conjugated antibodies against surface proteins for 20 minutes at 4°C, and then washed with fluorescence-activated cell sorting buffer (PBS + 1% FBS + 0.05% sodium azide). For intracellular cytokine staining, the cells stained with LIVE/DEAD and antibodies against surface proteins were fixed and permeabilized using a Foxp3/Transcription Factor Staining Buffer Set (Invitrogen), and then further stained with antibodies against intracellular proteins.

For the analysis of NK-cell subpopulations from PBMCs, singlet cells were gated first, followed by lymph gating. Positive cells for dump colors (Live/Dead, anti-CD14, anti-CD19, PE-Texas Red) were excluded. CD3–CD56+ cells were gated as NK cells and analyzed for the expression of CD16 and CD56. Consequently, the frequencies of conventional CD56+ (CD56dim), conventional CD56− CD16+ (cCD56bright), conventional CD56− CD16− (cCD56dim), and uCD56+ NK cells were determined among CD3–CD56+ NK cells. The expression of NK receptors, proliferation marker, and cytotoxic molecules within each NK subpopulation was also analyzed. Fluorescence minus one samples were used as negative controls. The gating strategies are presented in Fig E3 (NK-cell gating) and Fig E9 (surface markers and intracellular cytokine staining gating) in this article’s Online Repository at www.jacionline.org.

Flow cytometry was performed using an LSR II instrument and FACSDiva software (BD, Franklin Lakes, NJ), and the data were analyzed using FlowJo software (BD).

NK-cell cytotoxicity assay

NK cells isolated from cryopreserved/thawed PBMCs using the NK-cell isolation kit (Miltenyi Biotec) were used as effector cells. K562 cells were used as target cells; they were labeled with PKH26 (Sigma-Aldrich) and cocultured with isolated NK cells at various effector to target ratios in 96-well U-bottom plates. After 6 hours, cocultured cells were gathered, and TO-PRO-3-iodide (Invitrogen) was added at a final concentration of 0.5 μM. The cells were immediately analyzed by flow cytometry. The background TO-PRO-3-iodide staining was evaluated by staining PKH26-labeled K562 cells without coculture with NK cells after 6-hour incubation in medium. The percent-specific lysis for each coculture was calculated as the % TO-PRO-3-iodide+ cells in background subtracted from the % TO-PRO-3-iodide+ cells in each coculture.

Statistical analysis

Statistical analyses were performed using Prism software version 8.4.3 (GraphPad, GraphPad Software, San Diego, Calif). The Mann-Whitney U test was used in unpaired nonparametric comparisons, and the Wilcoxon matched-pairs signed rank test was used in paired nonparametric comparisons. The nonparametric Spearman rank correlation test was used to evaluate correlations between 2 parameters. The 2-way ANOVA test was used in group comparisons. P less than .05 was considered significant. In the analysis of next-generation sequencing (NGS) data, we used R program to implement a Wald test for significance testing (ie, the shrunk estimate of the logarithmic fold change divided by its SE). The Wald test P values were adjusted for multiple testing using the procedure of Benjamini and Hochberg.

RESULTS

Transcriptional profiles of NK cells from patients with COVID-19

We recruited 13 healthy donors, 7 patients with influenza, and 30 patients with COVID-19 (mild, n = 22; severe, n = 8) for this frequency among each NK-cell subset, and the expression of NGK2D is presented as the geometric MFI (gMFI). B, The expression of activation markers, including HLA-DR and CD25, was compared between cCD56dim (blue bar) and uCD56dim (red bar) NK cells from 21 patients with COVID-19. Left, Representative flow cytometry plots. Right, Bar plots showing cumulative data regarding the relative frequencies of CD4–CD25 in each NK-cell subset. C, Representative half-offset flow cytometry histograms (left) showing Ki-67, perforin, and granzyme B expression in each NK-cell subset. Bar plots (right) are cumulative data regarding Ki-67 expression (%), perforin, and granzyme B (gMFI) in each NK-cell subset (green for cCD56dim, blue for cCD56+, red for uCD56dim NK cells). Data from 8 healthy donors, 7 patients with influenza, 12 patients with mild COVID-19, and 7 patients with severe COVID-19 are presented. D, Assessment of NK-cell cytotoxicity the first week after diagnosis (9 healthy donors, 7 patients with mild COVID-19, and 5 patients with severe COVID-19). E, Correlation of the percentage of specific lysis at an E:T ratio of 10:1 and the frequency of uCD56+ NK cells. Data from 6 healthy donors, 3 patients with mild COVID-19, and 4 patients with severe COVID-19 are presented. E:T, Effector to target; KiR, inhibitory killer-cell immunoglobulin-like receptor; ns, not significant; TIGIT, T-cell immunoglobulin and ITIM domain. Error bars indicate SD. *P < .05, **P < .01, ***P < .0001.
FIG 4. The longitudinal change in NK cells. A and B, The longitudinal change in the frequencies of NK-cell subsets in 17 mild (Fig 4, A) and 8 severe (Fig 4, B) patients. Each representative flow cytometry plot of NK cells on the left is from 1 patient at 3 different time points after diagnosis. Right graphs represent cumulative data regarding the frequencies of each NK-cell subset (green for cCD56<sup>bright</sup>, blue for cCD56<sup>dim</sup>, and red for uCD56<sup>dim</sup> NK cells). Statistical analysis at each time point was a comparison to day-1 samples. C, Frequency of uCD56<sup>dim</sup> (left) and cCD56<sup>dim</sup> (right) at each time point between mild patients (blue line) and severe COVID-19 patients. 

D, E, F, Specific lysis of NK cells in 1<sup>st</sup> week, 2<sup>nd</sup> week, and since 3<sup>rd</sup> week after diagnosis, and GSVA enrichment score of cluster 1.
study. We used cryopreserved PBMCs from the patients with COVID-19 at several different time points. The clinical characteristics of the enrolled patients with COVID-19 are presented in Table E1, and the time points when samples were obtained are presented in Table E2.

First, we sorted CD3−CD56dim NK cells from cryopreserved PBMCs of healthy donors (n = 8) and patients with COVID-19 (mild, n = 13; severe, n = 8) and performed RNA-seq. When we analyzed the data obtained at the earliest time point for each patient with COVID-19, 217 genes (cluster 1) were upregulated and 208 genes (cluster 2) were downregulated in NK cells compared with the healthy donors (Fig 1, A and B). Cluster 1 was commonly enriched in previously reported gene sets related to SARS-CoV-2 infection (see Fig E2, A, in this article’s Online Repository at www.jacionline.org). The Kyoto Encyclopedia of Genes and Genomes pathway analysis revealed that cluster 1 was highly enriched in proinflammatory cytokine-responsive gene signatures from the LINCS L1000 data set. Cluster 1 was significantly associated with “cytokine-mediated signaling pathway,” also with “IkB kinase/NF-κB signaling,” “response to cytokine,” “cellular response to type I interferon,” and “type I interferon signaling pathway” in the gene ontology analysis of biological processes (Fig 1, D). However, Kyoto Encyclopedia of Genes and Genomes pathway and gene ontology analysis showed that cluster 2 was associated with several gene sets, including “HSV 1 infection,” “homologous recombination,” “telomere capping,” and “nonrecombinant repair” with relatively high P values (Fig E2, B and C).

To determine which cytokine contributed to these changes in the NK cells, we performed gene set enrichment analysis using cytokine-responsive gene signatures from the LINCS L1000 data set. Cluster 1 was highly enriched in proinflammatory cytokine-responsive gene signatures, including IL-1, TNF-α, and IL-6 (Fig 1, E). The protein-protein interaction network analysis revealed that key transcription factors associated with NK cells in COVID-19 are signal transducer and activator of transcription 3, signal transducer and activator of transcription 1, nuclear factor kappa B subunit 1, and tumor protein p53 (Fig 1, F).

**Expansion of the uCD56dim NK-cell population in patients with COVID-19**

To examine the phenotype of NK cells in patients with COVID-19, we performed flow cytometric analysis using cryopreserved PBMCs from healthy donors (n = 13), patients with severe influenza (n = 7), patients with mild COVID-19 (n = 17), and patients with severe COVID-19 (n = 8). Samples obtained on the day of diagnosis (day 1) were used for patients with influenza and patients with COVID-19. First, we analyzed the frequency of NK cells among CD14+CD19+ lymphocytes (Fig 2, A) and absolute number (Fig E4, A, in this article’s Online Repository at www.jacionline.org) and found no significant differences between the groups. We also found no significant difference in the frequency of NKG2C+CD57+ adaptive NK cells and CD3+CD56+ T cells between each group (see Figs E5 and E6 in this article’s Online Repository at www.jacionline.org).

To demonstrate the expansion of the uCD56dim NK-cell population during the early phase of COVID-19, we analyzed longitudinal PBMC samples obtained from 2 patients during the early acute phase with short intervals from the day of symptom onset. We found that the frequency of uCD56dim NK cells was low on the day of symptom onset and gradually increased during the first 4 days after symptom onset (Fig 2, E), indicating that the uCD56dim NK-cell population expands during the early phase of COVID-19.

We analyzed the NK-cell subpopulations using freshly isolated PBMC samples and matched cryopreserved PBMC samples and found that the uCD56dim NK-cell population was a minor population in both fresh and cryopreserved PBMC samples from healthy donors (see Fig E7, A, in this article’s Online Repository at www.jacionline.org). In patients with COVID-19, the uCD56dim NK-cell population was prominently observed in cryopreserved samples, but not in freshly isolated samples (Fig E7, B), indicating that the frequency of the uCD56dim NK-cell population was significantly increased by cryopreservation and thawing only in patients with COVID-19. These data show that NK cells from patients with COVID-19 easily lose their CD16 expression via cellular stress.

The expansion of uCD56dim NK-cell population was common in patients with COVID-19 regardless of clinical parameters, including sex, age, viral titers, white blood cell count, and C-reactive protein levels (see Fig E8 in this article’s Online Repository at www.jacionline.org).

**uCD56dim NK cells as a phenotypically and functionally distinct NK-cell subset**

To identify the characteristics of the uCD56dim NK-cell population, we compared the expression of activating receptors, including Nkp30, Nkp44, Nkp46, NKG2C, and NKGD2, and inhibitory receptors, including inhibitory killer-cell immunoglobulin-like receptors, T-cell immunoglobulin and ITIM domain, and NKGA2, using flow cytometry. The uCD56dim NK cells resembled CD56dim more than CD56bright with low expression of Nkp30, Nkp46, NKGA2, and NKGD2 and high expression of inhibitory killer-cell immunoglobulin-like receptors, T-cell immunoglobulin and ITIM domain, and NKGA2 (Fig 3, A).
However, uCD56\textsuperscript{dim} NK cells were distinguished from cCD56\textsuperscript{dim} NK cells by higher expression of NKp30, NKp46, and NKG2A and lower expression of inhibitory killer-cell immunoglobulin-like receptors, T-cell immunoglobulin and TIM domain, and NKG2C (Fig 3, A).

We also examined the expression of activation markers and found that HLA-DR and CD25 are upregulated in uCD56\textsuperscript{dim} NK cells compared with cCD56\textsuperscript{dim} NK cells, indicating that uCD56\textsuperscript{dim} NK cells were recently activated (Fig 3, B).

The cCD56\textsuperscript{dim} NK cells are cytolytic NK cells, and cCD56\textsuperscript{bright} NK cells are highly proliferating and cytokine-producing NK cells.\textsuperscript{11,25} Consistent with previous studies, cCD56\textsuperscript{dim} NK cells expressed a lower level of Ki-67 and higher levels of perforin and granzyme B compared with the cCD56\textsuperscript{bright} NK cells (Fig 3, C). However, uCD56\textsuperscript{dim} NK cells exhibited a distinctive functional phenotype from both cCD56\textsuperscript{dim} and cCD56\textsuperscript{bright} NK cells, with similar expression of Ki-67 but higher expression of perforin and granzyme B than in cCD56\textsuperscript{bright} NK cells, and higher expression of Ki-67 but lower expression of perforin and granzyme B than in cCD56\textsuperscript{dim} NK cells (Fig 3, C), indicating that uCD56\textsuperscript{dim} NK cells exhibit a higher proliferating capacity but lower cytotoxic capacity compared with cCD56\textsuperscript{dim} NK cells.

In addition, we examined the expression of CD62L, which is known to be cleaved by ADAM17 in activated NK cells,\textsuperscript{26} and found that the frequency of CD62L\textsuperscript{−} cells was significantly lower among uCD56\textsuperscript{dim} NK cells than among cCD56\textsuperscript{dim} NK cells (see Fig E10 in this article’s Online Repository at www.jacionline.org), indicating that ADAM17 activity is increased in uCD56\textsuperscript{dim} NK cells. Given that CD16 can also be cleaved by ADAM17,\textsuperscript{26,27} these results suggest that NK cells from patients with COVID-19 easily lose CD16 expression via increased ADAM17 activity.

**Impaired NK-cell cytotoxicity in patients with COVID-19**

Next, we evaluated the cytotoxic function of NK cells. We isolated NK cells from the PBMCs of healthy donors and patients with COVID-19 the first week after diagnosis and assessed the NK-cell cytotoxicity against K562 cells. We found that NK-cell cytotoxicity was significantly impaired in patients with COVID-19 compared with healthy donors regardless of disease severity (Fig 3, D). Furthermore, the NK-cell cytotoxicity negatively correlated with the relative frequency of uCD56\textsuperscript{dim} NK cells in the NK-cell population (Fig 3, E). Taken together, these results indicate that the expansion of the uCD56\textsuperscript{dim} subset, which had decreased expression of perforin and granzyme B compared with cCD56\textsuperscript{dim} NK cells, leads to impaired cytotoxicity of the NK-cell population in patients with COVID-19.

**Recovery of impaired NK-cell cytotoxicity and the cCD56\textsuperscript{dim} NK-cell population depending on disease severity**

Finally, we investigated how the expansion of uCD56\textsuperscript{dim} NK cells and impaired NK-cell cytotoxicity in patients with COVID-19 changes during the course of disease. We examined PBMCs collected from patients with COVID-19 at serial time points (indicated in Table E2) by flow cytometry and found that the frequency of uCD56\textsuperscript{dim} NK cells decreased more rapidly in mild patients than in severe patients (Fig 4, A and B). Until 7 days after diagnosis, we found no differences in the frequency of uCD56\textsuperscript{dim} and cCD56\textsuperscript{dim} NK cells between the 2 groups (Fig 4, C), but from 7 days after diagnosis, the frequency of uCD56\textsuperscript{dim} NK cells was significantly higher and the frequency of cCD56\textsuperscript{dim} NK cells was significantly lower in severe patients compared with mild patients (Fig 4, C). After 15 days from diagnosis, the differences disappeared, as the frequency of uCD56\textsuperscript{dim} NK cells decreased and the frequency of cCD56\textsuperscript{dim} NK cells increased in patients with severe COVID-19 (Fig 4, C).

We also performed combined t-distributed stochastic neighbor embedding analysis of NK cells from healthy donors, patients with influenza, and patients with mild and severe COVID-19 using flow cytometry data to examine the NK-cell population clusters and changes during the disease course (see Fig E11 in this article’s Online Repository at www.jacionline.org). The t-distributed stochastic neighbor embedding analysis revealed 6 different clusters of NK cells, and they could be grouped into 3 major populations: cCD56\textsuperscript{bright} NK cells, cCD56\textsuperscript{dim}CD16\textsuperscript{pos} NK cells, and cCD56\textsuperscript{dim}CD16\textsuperscript{neg} NK cells (Fig E11, A and B). Further analysis demonstrated that the cCD56\textsuperscript{dim}CD16\textsuperscript{neg} NK-cell population was exclusively expanded in patients with COVID-19, and it normalized rapidly in patients with mild COVID-19 (Fig E11, C-E).

In addition, we evaluated the NK-cell cytotoxicity during the course of disease. The second week after diagnosis, the impaired NK-cell cytotoxicity recovered in mild patients but not in severe patients (Fig 4, D and E). Since the third week after diagnosis, the impaired NK-cell cytotoxicity also recovered in severe patients, and the differences among disease groups disappeared (Fig 4, D and E). The results from the NK-cell cytotoxicity assays are compatible with the faster disappearance of uCD56\textsuperscript{dim} NK cells and faster reemergence of cCD56\textsuperscript{dim} NK cells in mild patients than in severe patients. We also analyzed RNA-seq data obtained from patients with COVID-19 (mild, n = 13; severe, n = 8) at serial time points. The gene set variation analysis enrichment score for cluster 1, upregulated genes in patients with COVID-19, decreased earlier in mild patients than in severe patients (Fig 4, F; see Fig E12 in this article’s Online Repository at www.jacionline.org), confirming the rapid and slow recovery of the NK-cell population in patients with mild and severe COVID-19, respectively.

**DISCUSSION**

Previous studies have reported that the absolute number of NK cells in patients with COVID-19 is reduced alongside lymphopenia, but the frequency of NK cells in lymphocytes is similar to that of healthy donors.\textsuperscript{17-19} Among NK cells, the frequency of CD56\textsuperscript{bright}CD16\textsuperscript{pos} NK cells has been reported to decrease in patients with COVID-19, especially those with severe disease, and the frequency of CD56\textsuperscript{dim}CD16\textsuperscript{pos} NK cells has been reported to slightly increase in patients with COVID-19. In the current study using cryopreserved PBMC samples, we found no difference in the frequency of NK cells among lymphocytes between healthy donors, patients with influenza, and patients with mild and severe COVID-19, and there was no difference in the frequency of cCD56\textsuperscript{bright} NK cells. Interestingly, the cCD56\textsuperscript{dim} NK-cell population was significantly reduced in patients with COVID-19 and the uCD56\textsuperscript{dim} NK-cell population was significantly expanded in patients with COVID-19 on the day of diagnosis regardless of severity, which was scarcely found in healthy donors and patients with influenza. The uCD56\textsuperscript{dim} NK...
cells had a higher proliferating capacity but lower cytolytic capacities than cCD56dim NK cells, which accounted for most of the NK cells in healthy donors and patients with influenza. The expansion of uCD56dim NK cells was associated with impaired NK-cell cytotoxicity in patients with COVID-19.

NK cells are a major subtype of type 1 innate lymphoid cells and play important roles against viral infections. Therefore, the study of NK-cell biology in viral diseases is important for understanding the natural disease course. However, because of the urgent need for the development of vaccines and treatments for COVID-19, more research and attention is focused on the adaptive immune responses, and understanding of the NK-cell biology in COVID-19 is lacking. In the present study, we aimed to identify the phenotypic and functional changes in NK cells during COVID-19 and how NK cells change during the course of the disease. We found that NK cells in COVID-19, regardless of disease severity, are characterized by the expansion of uCD56dim NK cells, which exhibit lower cytolytic capacities than do cCD56dim NK cells. This characteristic change in NK cells during COVID-19 was accompanied by impaired NK-cell cytotoxicity. Both the expansion of uCD56dim NK cells and impaired NK-cell cytotoxicity recovered earlier in mild patients compared with severe patients. A few studies have reported that the natural cytotoxicity of PBMCs from patients with COVID-19 is impaired, but to our knowledge, our study is the first to show the impaired function of NK cells directly using sorted CD3−CD56+ NK cells and how their function recovered during the course of the disease.

The expansion of the uCD56dim NK-cell population has been reported in a few clinical conditions, including malignancies, infectious mononucleosis, haploidentical hematopoietic stem cell transplantation, and hemophagocytic lymphohistiocytosis (HLH). However, the mechanism underlying the expansion of the uCD56dim NK-cell population has not been clarified. Previously, uCD56dim NK cells were considered to be the precursors of cCD56dim NK cells. However, several studies have shown that uCD56dim NK cells are bona fide differentiated NK cells. Interestingly, the loss of CD16 expression on NK cells by cryopreservation/thawing of human PBMCs has been reported in previous studies. The uCD56dim NK-cell population was prominently observed in cryopreserved PBMCs from patients with COVID-19, but not healthy donors, indicating that NK cells become abnormally labile to ex vivo cellular stress, such as cryopreservation/thawing, during COVID-19. In the present study, we also showed that the frequency of CD62L+ cells was significantly lower among uCD56dim NK cells than among cCD56dim NK cells (Fig E10), indicating that the ADAM17 activity is increased in uCD56dim NK cells. Given that CD16 can also be cleaved by ADAM17, these results suggest that NK cells from patients with COVID-19 easily lose CD16 expression via increased ADAM17 activity. In this regard, we hypothesize that ADAM17 activity is involved in the cleavage and loss of CD16 on NK cells in patients with COVID-19.

In addition, considering that COVID-19–specific upregulated genes were significantly enriched in gene sets regulated by proinflammatory cytokines, such as IL-1, TNF-α, and IL-6, we hypothesize that high levels of proinflammatory cytokines can be one of the mechanisms inducing the expansion of uCD56dim NK-cell population. The clinical conditions previously reported for the expansion of uCD56dim NK cells are commonly characterized by overwhelming inflammation and hypersecretion of various cytokines. However, the frequency of uCD56dim NK cells did not correlate with C-reactive protein levels in the current study. Further studies are required to reveal the origin and fate of uCD56dim NK cells.

HLH is a fatal systemic inflammatory syndrome and can be caused by several medical conditions, inducing strong activation of the immune system, such as viral infection or cancer (secondary HLH). Recently, several studies have reported that patients with severe COVID-19 undergo clinical conditions reminiscent of secondary HLH. In the current study, the expansion of uCD56dim NK cells lasted for a longer period in patients with severe COVID-19 than in patients with mild COVID-19. Particularly, in a mortality case, the frequency of uCD56dim NK cells remained elevated (>30%) during the course of the disease. Secondary HLH needs to be suspected in patients with COVID-19, particularly when the high frequency of uCD56dim NK cells is sustained.

**CONCLUSIONS**

We found that a unique NK-cell subset, uCD56dim NK cells, is expanded in COVID-19. We demonstrated that the NK-cell cytotoxicity was impaired in COVID-19 alongside the expansion of uCD56dim NK cells. Furthermore, we longitudinally showed that the rapid disappearance of uCD56dim NK cells and reemergence of cCD56dim NK cells in mild patients was accompanied by rapid recovery of NK-cell cytotoxicity. However, the recovery of the NK-cell population occurred in patients with severe COVID-19 in a delayed manner. To our knowledge, this was the first longitudinal study assessing NK cells from patients with COVID-19 in regard to phenotypic and functional changes. Further studies are needed on how these characteristic uCD56dim NK cells play a role in the protection or pathology of patients with COVID-19.

**Key messages**

- The uCD56dim NK-cell population expands in cryopreserved PBMCs from patients with COVID-19 regardless of disease severity.
- The expansion of uCD56dim NK cells is accompanied by decreased NK-cell cytotoxicity.
- The expansion of uCD56dim NK cells lasts longer in severe COVID-19 than in mild COVID-19.

**REFERENCES**

1. Wu F, Zhao S, Yu B, Chen YM, Wang W, Song ZG, et al. A new coronavirus associated with human respiratory disease in China. Nature 2020;579:265-9.
2. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med 2020;382:727-33.
3. World Health Organization. Weekly epidemiological update—19 January 2021. Available at: https://www.who.int/publications/m/item/weekly-epidemiological-update—19-january-2021. Accessed January 19, 2021.
4. Guan WJ, Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. Clinical characteristics of coronavirus disease 2019 in China. N Engl J Med 2020;382:1708-20.
5. Ferretti AP, Kula T, Wang Y, Nguyen DMV, Weinheimer A, Dunlap GS, et al. Unbiased screens show CD8(+) T cells of COVID-19 patients recognize shared epitopes in SARS-CoV-2 that largely reside outside the spike protein. Immunity 2020;53:1095-107.e3.
6. Gritton A, Weiskopf D, Ramirez SI, Mateus J, Dan JM, Modderbacher CR, et al. Targets of T cell responses to SARS-CoV-2 coronavirus in humans with COVID-19 disease and unexposed individuals. Cell 2020;181:1489-501.e15.
functions of natural killer cells: development, maturation, and clinical utilization. Front Immunol 2018;9:187-223.

Fano YY, Yang BY, Wu CY. Phenotypically and functionally distinct subsets of natural killer cells in human PBMCs. Cell Biol Int 2008;32:188-97.

Amand M, Iserentant G, Poli A, Sleiman M, Fievez V, Sanchez JP, et al. Human CD56(dim)CD16(dim) cells as an individualized natural killer cell subset. Front Immunol 2017;8:699.

Liu B, Han J, Cheng X, Yu L, Zhang L, Wang W, et al. Reduced numbers of T cells and B cells correlates with persistent SARS-CoV-2 presence in non-severe COVID-19 patients. Sci Rep 2020;10:17718.

Mazzoni A, Salvati L, Maggi L, Capone M, Vanni A, Spinacci M, et al. Impaired immune cell cytotoxicity in severe COVID-19 is IL-6 dependent. J Clin Invest 2020;130:4694-703.

Maucourtant C, Filipovic I, Ponzetta A, Aleman S, Cornillet M, Hertwig L, et al. Natural killer cell immunotypes related to COVID-19 disease severity. Sci Immunol 2020;5:eabd882.

Osman M, Faridi RM, Sigl W, Shabani-Rad MT, Dharmani-Khan P, Parker A, et al. Impaired natural killer cell counts and cytotoxic activity in patients with severe COVID-19. Blood Adv 2020;4:5035-9.

Varchetta S, Mele D, Oliviero B, Mantovani S, Ludovisi S, Cerino A, et al. Unique immunological profile in patients with COVID-19. Cell Mol Immunol 2021;18:604-12.

Dobin A, Davis CA, Schlesinger F, Drenkov K, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013;29:15-21.

Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 2014;15:550.

Chen EY, Tan CM, Kou Y, Duan Q, Wang Z, Vaz Meirelles G, et al. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinformatics 2013;14:128.

Kuleshov MV, Jones MR, Rouillard AD, Fernandez NF, Duan Q, Wang Z, et al. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Res 2016;44:W102-W107.

Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Statist Soc B 1995;57:289-300.

Frey Ag, Caligiuri MA. Human natural killer cell development. Immunol Rev 2006;214:239-50.

Vivier E, Tomasel E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol 2008;9:503-10.

Alessandro M, Cristina B, Massimo V, Daniela P, Claudia C, Maria Cristina M, et al. Activating receptors and coreceptors involved in human natural killer cell-mediated cytosis. Annu Rev Immunol 2001;19:197-223.

Fan YY, Yang BY, Wu CY. Phenotypically and functionally distinct subsets of natural killer cells in human PBMCs. Cell Biol Int 2008;32:188-97.

Amand M, Ierentant G, Poli A, Sleiman M, Fievez V, Sanchez JP, et al. Human CD56(dim)CD16(dim) cells as an individualized natural killer cell subset. Front Immunol 2017;8:699.

Liu B, Han J, Cheng X, Yu L, Zhang L, Wang W, et al. Reduced numbers of T cells and B cells correlates with persistent SARS-CoV-2 presence in non-severe COVID-19 patients. Sci Rep 2020;10:17718.

Mazzoni A, Salvati L, Maggi L, Capone M, Vanni A, Spinacci M, et al. Impaired immune cell cytotoxicity in severe COVID-19 is IL-6 dependent. J Clin Invest 2020;130:4694-703.

Maucourtant C, Filipovic I, Ponzetta A, Aleman S, Cornillet M, Hertwig L, et al. Natural killer cell immunotypes related to COVID-19 disease severity. Sci Immunol 2020;5:eabd882.

Osman M, Faridi RM, Sigl W, Shabani-Rad MT, Dharmani-Khan P, Parker A, et al. Impaired natural killer cell counts and cytotoxic activity in patients with severe COVID-19. Blood Adv 2020;4:5035-9.

Varchetta S, Mele D, Oliviero B, Mantovani S, Ludovisi S, Cerino A, et al. Unique immunological profile in patients with COVID-19. Cell Mol Immunol 2021;18:604-12.

Dobin A, Davis CA, Schlesinger F, Drenkov K, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013;29:15-21.

Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 2014;15:550.

Chen EY, Tan CM, Kou Y, Duan Q, Wang Z, Vaz Meirelles G, et al. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinformatics 2013;14:128.

Kuleshov MV, Jones MR, Rouillard AD, Fernandez NF, Duan Q, Wang Z, et al. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Res 2016;44:W102-W107.

Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Statist Soc B 1995;57:289-300.

Frey Ag, Caligiuri MA. Human natural killer cell development. Immunol Rev 2006;214:239-50.

Vivier E, Tomasel E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol 2008;9:503-10.

Alessandro M, Cristina B, Massimo V, Daniela P, Claudia C, Maria Cristina M, et al. Activating receptors and coreceptors involved in human natural killer cell-mediated cytosis. Annu Rev Immunol 2001;19:197-223.

Fan YY, Yang BY, Wu CY. Phenotypically and functionally distinct subsets of natural killer cells in human PBMCs. Cell Biol Int 2008;32:188-97.

Amand M, Ierentant G, Poli A, Sleiman M, Fievez V, Sanchez JP, et al. Human CD56(dim)CD16(dim) cells as an individualized natural killer cell subset. Front Immunol 2017;8:699.

Liu B, Han J, Cheng X, Yu L, Zhang L, Wang W, et al. Reduced numbers of T cells and B cells correlates with persistent SARS-CoV-2 presence in non-severe COVID-19 patients. Sci Rep 2020;10:17718.

Mazzoni A, Salvati L, Maggi L, Capone M, Vanni A, Spinacci M, et al. Impaired immune cell cytotoxicity in severe COVID-19 is IL-6 dependent. J Clin Invest 2020;130:4694-703.

Maucourtant C, Filipovic I, Ponzetta A, Aleman S, Cornillet M, Hertwig L, et al. Natural killer cell immunotypes related to COVID-19 disease severity. Sci Immunol 2020;5:eabd882.

Osman M, Faridi RM, Sigl W, Shabani-Rad MT, Dharmani-Khan P, Parker A, et al. Impaired natural killer cell counts and cytotoxic activity in patients with severe COVID-19. Blood Adv 2020;4:5035-9.

Varchetta S, Mele D, Oliviero B, Mantovani S, Ludovisi S, Cerino A, et al. Unique immunological profile in patients with COVID-19. Cell Mol Immunol 2021;18:604-12.
REFERENCES

E1. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013;29:15-21.

E2. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 2014;15:550.
FIG E1. Gating strategies for NK-cell sorting before RNA-seq. After singlets and lymphocytes were gated by FSC and SSC, live cells were gated by 7-AAD staining. The CD3–CD56+ cells were then gated for NK cells. FSC-A, forward scatter-area; FSC-H, forward scatter-height; SSC-A, side scatter-area.
FIG E2.  Gene set enrichment analysis of clusters 1 and 2. A, Bar graphs showing the top 10 enriched gene sets for cluster 1 from published COVID-19–related gene sets. GSE numbers are indicated. B and C, KEGG pathway analysis (Fig E2, B) and GO biological processes analysis with GO IDs (Fig E2, C) for cluster 2. The dashed line in Fig E2, B, indicates a P value of .05. The enriched pathways with P less than .05 are presented as red bars, and others are presented as gray bars. GO, Gene ontology; GSE, gene set enrichment; KEGG, Kyoto Encyclopedia of Genes and Genomes; MOI, multiplicity of infection; HSV1, herpes simplex virus type 1; ECM, extracellular matrix.
FIG E3. Gating strategies for flow cytometric analysis of CD56 and CD16. FMO, Fluorescence minus one; FSC-A, forward scatter-area; FSC-H, forward scatter-height; SSC-A, side scatter-area.
FIG E4. Comparison of the absolute number of NK cells among patients with influenza and patients with mild and severe COVID-19. All data are presented as absolute counts of NK cells in 1 mL of blood. A, Comparison of the absolute number of NK cells among disease groups. B, Comparison of the absolute number of NK cells in each subset among disease groups. Blue dots represent patients with influenza, orange dots represent patients with mild COVID-19, and red dots represent patients with severe COVID-19. ns, Not significant. *P < .05.
FIG E5. The frequency of adaptive NK cells in patients with mild and severe COVID-19. Left, Representative flow cytometry plots showing the gating strategy for adaptive NK cells (CD57\(^+\)NKG2C\(^+\)CD56\(^{dim}\) NK cells) in PBMCs. Right, Bar plots showing cumulative data regarding the frequency of adaptive NK cells in patients with mild COVID-19 (orange bar, \(n = 14\)) and patients with severe COVID-19 (red bar, \(n = 7\)). ns, Not significant.
**FIG E6.** The frequency of CD3⁺CD56⁺ cells in each disease group. **Left,** Representative flow cytometry plots showing the gating strategy for CD3⁺CD56⁺ cells in PBMCs. **Right,** Bar plots showing cumulative data regarding the frequency of CD3⁺CD56⁺ cells among CD14⁻CD19⁻ lymphocytes in healthy donors (green bar, n = 8), patients with influenza (blue bar, n = 7), patients with mild COVID-19 (orange bar, n = 14), and patients with severe COVID-19 (red bar, n = 8). **FSC-A,** forward scatter-area; **FSC-H,** forward scatter-height; **ns,** not significant; **SSC-A,** side scatter-area.
**FIG E7.** Comparison of NK-cell subsets between freshly isolated PBMCs and cryopreserved PBMCs from healthy donors (A) and patients with COVID-19 (B). Left, Representative flow cytometry plots. Right, Bar graph showing cumulative data regarding the paired comparison of the frequency of the uCD56<sup>dim</sup> subset among NK cells in healthy donors (Fig E7, A) and patients with COVID-19 (Fig E7, B). *ns*, Not significant. *P < .05.*
FIG E8. Correlation between the expansion of uCD56dim NK-cell population and clinical parameters. Each bar plot and dot plot represents the correlation between the frequency of uCD56dim NK cells in patients with COVID-19 and clinical parameters, including sex (A), age (B), viral titers (C), WBC counts (D), and CRP (E). N = 25 (17 patients with mild COVID-19 and 8 patients with severe COVID-19). CRP, C-reactive protein; WBC, white blood cell.
FIG E9. Gating strategies for flow cytometric analysis of surface markers and intracellular cytokine staining. Flow cytometry plots for the staining of each marker (red line) in uCD56<sup>dim</sup> NK cells. Fluorescence minus one (FMO; gray bar) was used as a negative control. Gray dashed lines are cutoff points determined by FMO staining. FSC-A, forward scatter-area; FSC-H, forward scatter-height; SSC-A, side scatter-area.
FIG E10. The expression of CD62L in CD56dim NK-cell subpopulations. Left, Representative flow cytometry plot from 1 patient. Right, Bar plots showing cumulative data regarding the relative frequency of CD62L in each NK-cell subset (green bar for cCD56bright, blue bar for cCD56dim, and red bar for uCD56dim; n = 21). Error bars indicate SD. ****P < .0001.
FIG E11. t-SNE analysis of NK cells. A-C, The t-SNE analysis of NK cells from 8 healthy donors, 7 patients with influenza, 8 patients with mild COVID-19, and 7 patients with severe COVID-19 on the day of diagnosis. A, The left t-SNE plot shows 6 different clusters, and the bar graph shows the normalized percentage of each cluster. B, Heatmap showing the differential expression of CD56, CD16, NKP30, NKP46, KIRs, and TIGIT in each group. C, The t-SNE analysis performed separately by disease group. The bar graph shows the normalized percentage of each cluster by group (green box for healthy donors, blue box for patients with influenza, orange box for patients with mild COVID-19, and red box for patients with severe COVID-19). D and E, The t-SNE analyses were performed separately by group (6 healthy donors, 5 patients with mild COVID-19, and 6 patients with severe COVID-19) 1 week after diagnosis (Fig E11, D) and 2 weeks after diagnosis (Fig E11, E). KIR, Inhibitory killer-cell immunoglobulin-like receptor; TIGIT, T-cell immunoglobulin and ITIM domain; t-SNE, t-distributed stochastic neighbor embedding.
FIG E12. Transcriptomic profiles of NK cells during the disease course by patient. 

**A**, Heatmap showing the dynamic changes in the expression of cluster 1 and cluster 2 genes during the course of the disease by patient. Patient ID is annotated in green, blue, and red boxes, and the time points are annotated below. 

**B**, The longitudinal GSVA enrichment score for cluster 1. The same patients are connected by a blue (patients with mild COVID-19) or red line (patients with severe COVID-19). 

GSVA, Gene set variation analysis.
| ID  | Age (y) | Sex | Disease severity | Mortality | Underlying diseases | Oxygen demand (L/min or oxygen % for MV) | Systolic BP | Respiratory rate (/min) | Mental status | ICU admission |
|-----|---------|-----|-----------------|-----------|---------------------|------------------------------------------|-------------|-----------------------|--------------|---------------|
| 1   | 46      | F   | 3               | 0         | DM, SCZ             | 0                                        | 96          | 22                    | A            | No            |
| 2   | 39      | F   | 3               | 0         | None                | 0                                        | 111         | 20                    | A            | No            |
| 3   | 31      | M   | 2               | 0         | None                | 0                                        | 151         | 18                    | A            | No            |
| 4   | 47      | F   | 2               | 0         | None                | 0                                        | 111         | 20                    | A            | No            |
| 5   | 53      | F   | 3               | 0         | None                | 2                                        | 143         | 26                    | A            | No            |
| 6   | 49      | M   | 3               | 0         | None                | 0                                        | 146         | 20                    | A            | No            |
| 7   | 55      | F   | 2               | 0         | HTN, DM             | 0                                        | 158         | 20                    | A            | No            |
| 8   | 86      | F   | 1               | 0         | HTN, A-fib, dementia| 0                                        | 107         | 20                    | A            | No            |
| 9   | 84      | M   | 3               | 0         | HTN, DM, dementia   | 4                                        | 129         | 20                    | A            | No            |
| 10  | 58      | M   | 3               | 0         | None                | 0                                        | 123         | 20                    | A            | No            |
| 11  | 59      | F   | 3               | 0         | None                | 0                                        | 121         | 20                    | A            | No            |
| 12  | 23      | M   | 2               | 0         | None                | 0                                        | 108         | 20                    | A            | No            |
| 13  | 51      | M   | 3               | 0         | None                | 0                                        | 114         | 18                    | A            | No            |
| 14  | 25      | M   | 2               | 0         | None                | 0                                        | 118         | 20                    | A            | No            |
| 15  | 49      | F   | 3               | 0         | HTN                 | 0                                        | 111         | 20                    | A            | No            |
| 16  | 22      | M   | 1               | 0         | None                | 0                                        | 115         | 20                    | A            | No            |
| 17  | 18      | F   | 1               | 0         | None                | 0                                        | 96          | 18                    | A            | No            |
| 18  | 24      | M   | 2               | 0         | None                | 0                                        | 101         | 18                    | A            | No            |
| 19  | 24      | F   | 1               | 0         | None                | 0                                        | 116         | 18                    | A            | No            |
| 20  | 61      | M   | 2               | 0         | None                | 0                                        | 118         | 20                    | A            | No            |
| 21  | 49      | M   | 1               | 0         | None                | 0                                        | 100         | 20                    | A            | No            |
| 22  | 29      | M   | 1               | 0         | None                | 0                                        | 112         | 20                    | A            | No            |
| 23  | 65      | M   | 5               | 0         | HTN, DM             | MV 70%                                   | 127         | 20                    | V            | Yes           |
| 24  | 63      | M   | 5               | 0         | SCZ                 | MV 40%                                   | 106         | 19                    | P            | Yes           |
| 25  | 64      | M   | 5               | 0         | HTN, SCZ            | MV 50%                                   | 127         | 20                    | P            | Yes           |
| 26  | 36      | M   | 5               | 0         | None                | MV 40%                                   | 143         | 20                    | A            | Yes           |
| 27  | 64      | F   | 5               | 0         | None                | MV 50%                                   | 102         | 26                    | U            | Yes           |
| 28  | 63      | F   | 5               | 0         | None                | MV 60%                                   | 96          | 28                    | A            | Yes           |
| 29  | 78      | F   | 5               | 1         | HTN                 | MV 100%                                  | 89          | 30                    | P            | Yes           |
| 30  | 41      | M   | 5               | 0         | None                | MV 60%                                   | 128         | 22                    | U            | Yes           |
| H1  | 31      | M   | —               | —         | None                | —                                        | —           | —                     | —            | —             |
| H2  | 31      | M   | —               | —         | None                | —                                        | —           | —                     | —            | —             |
| H3  | 38      | M   | —               | —         | None                | —                                        | —           | —                     | —            | —             |
| H4  | 38      | M   | —               | —         | None                | —                                        | —           | —                     | —            | —             |
| H5  | 30      | M   | —               | —         | None                | —                                        | —           | —                     | —            | —             |
| H6  | 27      | M   | —               | —         | None                | —                                        | —           | —                     | —            | —             |
| H7  | 28      | F   | —               | —         | None                | —                                        | —           | —                     | —            | —             |
| H8  | 36      | M   | —               | —         | None                | —                                        | —           | —                     | —            | —             |
| H9  | 35      | M   | —               | —         | None                | —                                        | —           | —                     | —            | —             |
| H10 | 45      | M   | —               | —         | None                | —                                        | —           | —                     | —            | —             |
| H11 | 55      | M   | —               | —         | None                | —                                        | —           | —                     | —            | —             |
| H12 | 27      | M   | —               | —         | None                | —                                        | —           | —                     | —            | —             |
| H13 | 36      | M   | —               | —         | None                | —                                        | —           | —                     | —            | —             |

| I1  | 89      | M   | 4               | 1         | Prostate cancer     | 2                                        | 98          | 18                    | A            | Yes           |
| I2  | 66      | M   | 5               | 0         | MM, HTN             | 10                                       | 103         | 24                    | V            | Yes           |
| I3  | 75      | F   | 2               | 0         | HTN, DM             | 1                                        | 130         | 24                    | A            | No            |
| I4  | 70      | M   | 3               | 1         | HTN, DM             | 0                                        | 120         | 16                    | A            | Yes           |
| I5  | 33      | M   | 2               | 0         | PCOS                | 0                                        | 134         | 20                    | A            | No            |
| I6  | 59      | M   | 3               | 0         | ESRD                | 0                                        | 144         | 18                    | A            | No            |
| I7  | 59      | F   | 3               | 0         | BLL                 | 0                                        | 118         | 18                    | A            | No            |

* A-fib: Atrial fibrillation; BLL: B-cell lymphocytic leukemia; BP: blood pressure; RR: respiratory rate; DM: diabetes mellitus; ESRD: end-stage renal disease; F: female; HTN: hypertension; ICU: intensive care unit; M: male; MM: multiple myeloma; MV: mechanical ventilation; NIH: National Institutes of Health; PCOS: polycystic ovary syndrome; SCZ: schizophrenia.

**Disease severity was calculated on the basis of NIH severity score (1 = asymptomatic, 2 = mild, 3 = moderate [pneumonia saturation ≥ 94%], 4 = severe [pneumonia with RR > 30, saturation < 94%, PaO2/FiO2 < 300, lung infiltration > 50%], 5 = critical [respiratory failure, shock, multiple organ dysfunction]).**

†Mortality is presented as 0 = alive and 1 = death.

§Mental status is presented as A = alert, V = voice, P = pain, and U = unresponsive.

§Mild and severe patients were classified by the NIH severity score (1-3 = mild, 4-5 = severe).
# TABLE E2. Sample time points and assessment assays

| Patient ID | Sampling time points* | RNA-seq | Immunophenotyping/ICS | Cytotoxicity assay |
|------------|-----------------------|---------|-----------------------|--------------------|
| **Mild patients** | | | | |
| 1 | 1, 3, 5, 7, 13 | 1, 7, 13 | 1, 3, 5, 7, 13 | |
| 2 | 1, 3, 5, 7, 14 | 1, 7, 14 | 1, 3, 5, 7, 14 | |
| 3 | 1, 3, 5, 7, 15 | 1, 7, 15 | 1, 3, 5, 7, 15 | |
| 4 | 1, 3, 5, 7, 15 | 1, 3, 5, 7, 15 | | |
| 5 | 1, 3, 5, 13, 17 | 5, 13, 17 | 1, 3, 13, 17 | 1 |
| 6 | 1, 3, 10, 17 | 3, 10, 17 | 1, 3, 10, 17 | 17 |
| 7 | 1, 3, 7, 14, 21 | 1, 7, 14 | 1, 3, 7, 14, 21 | |
| 8 | 1, 3, 7, 14 | 1, 7, 14 | 1, 3, 7, 14 | |
| 9 | 1, 3, 7, 14 | 1, 7, 20 | 1, 3, 7, 14 | |
| 10 | 1, 3, 7 | 1, 7, 20 | 1, 3, 7, 14 | |
| 11 | 1, 3, 7, 14 | 1, 7, 14 | 1, 3, 7, 14 | |
| 12 | 1, 3, 7, 14 | 1, 7, 14 | 1, 3, 7, 14 | |
| 13 | 1, 7, 14 | 1, 7, 14 | 1, 3, 7, 14 | |
| 14 | 1, 7, 21 | 1, 7, 21 | 1, 7, 14 | |
| 15 | 7, 21 | | 7, 21 | |
| 16 | 1, 7, 14 | 1, 7, 14 | 1, 7 | |
| 17 | 1, 7, 14 | 1, 7, 14 | 1, 7 | |
| 18 | 1, 7, 14 | 1, 7, 14 | 1, 7 | |
| 19 | 1, 7 | 1, 7 | 1, 7 | |
| 20 | 1, 7, 21 | 1, 7, 21 | 1, 7 | |
| 21 | 1, 7, 21 | 1, 7, 21 | 1, 7 | |
| 22 | 1, 7 | 1, 7 | 1, 7 | |
| **Severe patients** | | | | |
| 23 | 1, 3, 5, 14, 17, 23 | 5, 17, 23 | 1, 5, 17, 23 | 3, 14, 23 |
| 24 | 1, 3, 5, 14, 17, 23 | 1, 5, 14, 17, 23 | 1, 5, 14, 17, 23 | |
| 25 | 1, 3, 5, 10, 14, 23, 26 | 5, 10, 24 | 1, 5, 10, 24 | 7, 31 |
| 26 | 1, 3, 5, 10, 24, 31 | 3, 7, 23 | 3, 7, 14, 23 | 3, 7, 23 |
| 27 | 1, 3, 5, 10, 24 | 5, 10, 24 | 1, 5, 10, 24 | 3, 24 |
| 28 | 1, 3, 5, 13, 17, 24 | 5, 13, 24 | 1, 3, 5, 13, 17, 24 | 3 |
| 29 | 1, 7, 10, 14, 18, 27, 32 | 10, 18, 27 | 1, 7, 14, 32 | 7 |
| 30 | 1, 3, 5, 7, 10, 19, 26, 30 | 5, 10, 19, 26 | 1, 3, 5, 7, 10, 19, 26, 30 | 7, 26 |

*ICS: Intracellular cytokine staining; NIH, National Institutes of Health.

*Sampling time points are presented as days after hospitalization.

†Assessment assays are presented as number of sampling time points.

‡Mild and severe patients are classified by NIH severity score (score 1-3: mild, 4-5: severe).
| Patient’s ID | Viral titer (Ct value) | WBC count (/μL) | Neutrophil (%) | Lymphocyte (%) | CRP (mg/dL) | Chest X-ray abnormalities * |
|--------------|------------------------|-----------------|----------------|----------------|-------------|---------------------------|
| 1           | Negative               | 12,000          | 83.4           | 10.5           | 5.5         | 1                         |
| 2           | 19.4                    | 4,500           | 82.7           | 13.2           | 0.5         | 0                         |
| 3           | 26.9                    | 8,430           | 59.8           | 26.5           | 0.6         | 0                         |
| 4           | 21.5                    | 4,360           | 72.2           | 19.3           | 0.3         | 0                         |
| 5           | 26.4                    | 4,770           | 65.5           | 24.7           | 0.7         | 0                         |
| 6           | 21.6                    | 3,920           | 63.2           | 23.5           | 0.8         | 1                         |
| 7           | 27.6                    | 13,300          | 67.2           | 24.9           | 0.3         | 0                         |
| 8           | 22.2                    | 3,800           | 51.4           | 36.7           | 1           | 0                         |
| 9           | 22.1                    | 9,500           | 76.2           | 10.8           | 15.5        | 1                         |
| 10          | Negative                | 4,600           | 77.3           | 15.4           | 1.7         | 1                         |
| 11          | 27.5                    | 7,300           | 82.9           | 14.3           | 0.3         | 0                         |
| 12          | 19.8                    | 3,880           | 69             | 23.5           | 0.3         | 0                         |
| 13          | 31.5                    | 6,560           | 57.4           | 32.8           | 5.1         | 1                         |
| 14          | 9.87                    | 4,630           | 48.6           | 35             | 0.3         | 0                         |
| 15          | 32.2                    | 5,540           | 70.3           | 23.6           | 0.3         | 0                         |
| 16          | 29.9                    | 3,710           | 51.8           | 39.1           | 0.5         | 0                         |
| 17          | 15.2                    | 2,700           | 39.3           | 52.6           | 0.3         | 0                         |
| 18          | 35.3                    | 6,310           | 46.4           | 39.9           | 0.5         | 0                         |
| 19          | 32.3                    | 5,450           | 66.9           | 26.5           | 0.5         | 0                         |
| 20          | 28.7                    | 8,400           | 79.2           | 12.4           | 2.1         | 0                         |
| 21          | Negative                | 5,600           | 64.1           | 28.7           | 0.6         | 0                         |
| 22          | 35.3                    | 6,230           | 51.9           | 37.9           | 0.8         | 0                         |
| Severe patients† |                  |                |                |                |             |                           |
| 23          | 20.8                    | 5,420           | 62.9           | 25.3           | 4.8         | 1                         |
| 24          | 33.0                    | 2,800           | 74.9           | 20.6           | 12.4        | 1                         |
| 25          | 24.2                    | 5,460           | 77.2           | 12.5           | 9.9         | 1                         |
| 26          | 18.8                    | 6,560           | 67.2           | 16.9           | 2.3         | 1                         |
| 27          | 29.8                    | 3,080           | 68.9           | 24             | 7.4         | 1                         |
| 28          | 34.8                    | 9,020           | 71.4           | 21.4           | 11.4        | 1                         |
| 29          | 25.1                    | 11,760          | 88.7           | 8.1            | 8.4         | 1                         |
| 30          | 19.1                    | 4,200           | 64.5           | 29.3           | 5.2         | 1                         |
| Patients with influenza |                  |                |                |                |             |                           |
| 11          | NA                      | 11,400          | 75.6           | 10.5           | 9.6         | 1                         |
| 12          | NA                      | 2,700           | 80             | 8.9            | 1.1         | 1                         |
| 13          | NA                      | 9,000           | 69.4           | 23.8           | 0.1         | 0                         |
| 14          | NA                      | 15,400          | 83.2           | 4.9            | 6.7         | 1                         |
| 15          | NA                      | 2,300           | 55.8           | 30.5           | 3.0         | 0                         |
| 16          | NA                      | 5,500           | 74.4           | 16.9           | 0.2         | 1                         |
| 17          | NA                      | 3,700           | 35.3           | 51.1           | 1.2         | 1                         |

CRP, C-reactive protein; NA, not available; NIH, National Institutes of Health; WBC, white blood cell.

*Chest abnormalities are presented as 0 = normal and 1 = abnormal.
†Mild and severe patients are classified by NIH severity score (1-3 = mild, 4-5 = severe).
### TABLE E4. Key resources

| Reagent or resource | Source | Identifier |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| BV421 anti-NKG2D (Clone: 1D11) | Biolegend | Cat# 320822 |
| BV510 anti-CD3 (Clone: HIT3a) | BD Biosciences | Cat# 564713 |
| BV605 anti-NKp44 (Clone: p44-8) | BD Biosciences | Cat# 744301 |
| BV605 anti-Ki-67 (Clone: Ki-67) | Biolegend | Cat# 350522 |
| BV650 anti-NKG2A (Clone: 131411) | BD Biosciences | Cat# 747920 |
| BV711 anti-NKG2C (Clone: 134591) | BD Biosciences | Cat# 748164 |
| FITC anti-KIR2D (Clone: REA1042) | Miltenyi Biotec | Cat# 130-117-477 |
| FITC anti-KIR3DL1/3DL2 (Clone: REA970) | Miltenyi Biotec | Cat# 130-116-177 |
| FITC anti-PERforin (Clone: dG9) | Biolegend | Cat# 308104 |
| FITC anti-CD3 (Clone: HIT3a) | BD Biosciences | Cat# 555339 |
| PerCP-cy5.5 anti–Granzyme B (Clone: QA16A02) | Biolegend | Cat# 372212 |
| PerCP-cy5.5 7-AAD (Clone: 8-AAD) | BD Biosciences | Cat# 51-68981E |
| PE anti-CD56 (Clone: HCD56) | Biolegend | Cat# 318306 |
| PE-TR anti-CD14 (Clone: MOP9) | BD Biosciences | Cat# 562335 |
| PE-TR anti-CD19 (Clone: HIB19) | BD Biosciences | Cat# 562294 |
| PE-Cy7 anti-TIGIT (Clone: MBSA43) | eBioscience | Cat# 25-9500-42 |
| AF647 anti-NKp30 (Clone: p30-15) | BD Biosciences | Cat# 558408 |
| AF700 anti-NKp46 (Clone: 900) | Biolegend | Cat# 331932 |
| APC-H7 anti-CD16 (Clone: 3G8) | BD Biosciences | Cat# 560195 |

**Critical commercial assays**

| Reagent or resource | Source | Identifier |
|---------------------|--------|------------|
| LIVE/DEAD Fixable Red Dead Cell Stain Kit | Invitrogen | Cat# L34972 |
| Foxp3/Transcription Factor Staining Buffer Set | Invitrogen | Cat# 00-5523-00 |
| PKH26 Red Fluorescent Cell Linker Kit | Sigma-Aldrich | Cat# PKH26GL |
| TO-PRO-3 Iodide | Invitrogen | Cat# T3605 |
| TRIzol LS reagent | Invitrogen | Cat# 10296028 |
| Lymphocyte Separation Medium | Corning | Cat# 25-072-CV |
| NK Cell Isolation Kit, human | Miltenyi Biotec | Cat# 130-092-657 |

**Deposited data**

| Raw and analyzed data | Genome Reference Consortium | http://www.ncbi.nlm.nih.gov/projectsgenome/assembly/grc/human/ |
|-----------------------|-----------------------------|---------------------------------------------------------------|
| Human reference genome GRCh38 | Genome Reference Consortium | http://www.ncbi.nlm.nih.gov/projects genome/assembly/grc/human/ |

**Experimental models: Cell lines**

| Reagent or resource | Source | Identifier |
|---------------------|--------|------------|
| K562 | American Type Culture Collection (ATCC) | ATCC CCL243 |

**Software and algorithms**

| Reagent or resource | Source | Identifier |
|---------------------|--------|------------|
| STAR | Dobin et al.11 2013 | http://doi:10.1093/bioinformatics/bts635 |
| DESeq2 | Love et al.12 2014 | http://doi:10.1186/s13059-014-0550-8 |
| Prism software version 8.4.3 | GraphPad | NA |
| FlowJo software version 10.7.1 | Treestar | https://www.flowjo.com/solutions/flowjo/downloads |
| FACSDiva software | BD Biosciences | NA |

**FITC**, Fluorescein isothiocyanate; **GEO**, Gene Expression Omnibus; **KIR**, inhibitory killer-cell immunoglobulin-like receptor; **NA**, not available; **TIGIT**, T-cell immunoglobulin and ITIM domain.
| Viability information for all samples after thawing | Patients with influenza* | Patients with mild COVID-19† | Patients with severe COVID-19§ |
|---|---|---|---|
| Viability, mean ± SD | 86.88 ± 7.35 | 69.21 ± 13.77 | 79.75 ± 12.11 | 75.22 ± 10.19 |

Separate information about the viability of samples is presented below (presented as Pt ID, time point, viability [%]).

*Healthy: H1, 1, 85.1; H2, 1, 77.0; H3, 1, 84.6; H4, 1, 77.6; H5, 1, 85.3; H6, 1, 83.0; H7, 1, 86.7; H8, 1, 83.0; H9, 1, 99.4; H10, 1, 99.2; H11, 1, 99.1; H12, 1, 82.5; H13, 1, 86.9.
†Patients with influenza: I1, 1, 92.2; I2, 1, 84.9; I3, 1, 84.9; I4, 1, 84.9; I5, 1, 84.9; I6, 1, 84.9; I7, 1, 84.9; I8, 1, 84.9; I9, 1, 84.9.
§Patients with severe COVID-19: 23, 1, 87.4; 23, 3, 78.8; 23, 7, 56.7; 23, 14, 81.9; 23, 17, 85.7; 23, 23, 83.4; 24, 1, 72.1; 24, 5, 64.2; 24, 8, 83.4; 24, 14, 86.5; 24, 23, 87.2; 24, 26, 57.0; 25, 1, 68.8; 25, 5, 74.9; 25, 7, 77.0; 25, 10, 72.3; 25, 25, 74.5; 25, 31, 63.8; 26, 1, 83.1; 26, 3, 78.3; 26, 7, 80.3; 26, 14, 74.7; 26, 23, 86.0; 27, 1, 59.6; 27, 3, 62.8; 27, 5, 61.8; 27, 10, 69.2; 27, 20, 64.3; 27, 24, 75.2; 28, 1, 85.4; 28, 3, 71.2; 28, 5, 73.3; 28, 7, 71.6; 28, 13, 84.1; 28, 17, 80.1; 28, 24, 79.6; 29, 1, 67.5; 29, 7, 45.4; 29, 14, 84.1; 29, 32, 83.5; 30, 1, 84.5; 30, 3, 91.2; 30, 5, 82.2; 30, 7, 67.7; 30, 10, 86.9; 30, 19, 92.9; 30, 26, 79.3; 30, 30, 81.0.