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Original Research

Natural Killer Cells in SARS-CoV-2-Vaccinated Subjects with Increased Effector Cytotoxic CD56^{dim} Cells and Memory-Like CD57^{+}NKG2C^{+}CD56^{dim} Cells

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Abstract

Background: The infection and negative effects of the SARS-CoV-2 (severe acute respiratory syndrome coronavirus) virus are mitigated by vaccines. It is unknown whether vaccination has worked by eliciting robust protective innate immune responses with high affinity. **Methods**: Twenty healthy volunteers received three doses of Comirnaty (Pfizer Australia Pty Ltd.) and were evaluated 9 months after the second vaccination and 1 month after the booster dose. The exclusion criteria were the presence of adverse effects following the vaccination, a history of smoking, and heterologous immunization. The inclusion criteria were the absence of prior Coronavirus Disease (COVID)-19 history, the absence of adverse effects, and the absence of comorbidities. Specific phenotype and levels of CD107a and granzyme production by blood NK (natural killer) cells were analyzed after exposure to SARS-CoV-2 spike antigen (Wuhan, Alpha B.1.1.7, Delta B.1.617.2, and Omicron B1.1.529 variants), and related with anti-SARS-CoV-2 antibody production. **Results**: The booster dose caused early NK CD56^{dim} subset activation and memory-like phenotype. **Conclusions**: We report the relevance of the innate immune response, especially NK cells, to SARS-CoV-2 vaccines to guarantee efficient protection against the infection following a booster dose.

Keywords: SARS-CoV-2; vaccine; innate response; NK cell

1. Introduction

The severe acute respiratory syndrome coronavirus, sometimes known as SARS-CoV-2 (severe acute respiratory syndrome coronavirus), is vaccine-preventable [1]. High affinity and persistent protective antibody responses indicate an efficient humoral immune response to vaccination [2]. By six months, there is a significant reduction in antibody responses, especially after vaccinations against SARS-CoV-2 mRNA [3].

A recent investigation found that the protection against Coronavirus Disease (COVID)-19-related hospitalization and death started to decrease in older adults and patients with weak or numerous medical conditions after 20 weeks [4]. Natural killer (NK) cells are essential for antiviral immunity. Virally infected host cells can be killed by NK cells by triggering apoptosis in various ways. Two of the proteins that it can first exocytose are perforin and granzymes, and when they interact, they can cause the target cell to undergo apoptosis [5]. By expressing the executioner molecules Fas cell surface death receptor (FAS) ligand (FasL) [6] and Tumor Necrosis Factor (TNF)-related apoptosis-inducing ligand (TRAIL) [7,8], NK cells can destroy the target. This stimulates the signaling of the extrinsic apoptotic pathway. NK cells can also release chemokines and pro-inflammatory cytokines such as Interferon (IFN)-gamma, TNF-beta, and granulocyte-macrophage colony-stimulating factor (GM-CSF) [9]. By encouraging pro- or anti-inflammatory tendencies as a disease progresses, NK cells exhibit functional plasticity [9].

Peripheral blood contains about 10–15% of human NK cells, which are recognized phenotypically by the presence of CD56 and CD16 on their surfaces and the absence of CD3. Based on the surface expression of CD56 and CD16, two subgroups of NK cells have been discovered in humans: CD56(+)^{bright}/CD16(-) cells and CD56(+)^{bright}/CD16(dim) cells. These categories are unique from one another in terms of function and homing abilities, in addition to physical characteristics. While CD56(+)^{bright}/CD16(-) cells are mainly found in lymph nodes and inflammatory areas, CD56(+)^{dim}/CD16(+)^{bright} cells are principally cytotoxic and detected in peripheral blood [10].

Increased evidence suggests that the SARS-CoV-2 infection may affect the tissue distribution and effector capabilities of NK cells and that a rapid NK cell response may determine a patient's clinical outcome. However, more research is required to pinpoint the precise role of NK cells in the pathophysiology of COVID-19. Patients who have SARS-CoV-2 infection experience lymphopenia as a symptom. In patients with severe infections, neutrophils and monocytopenia frequently coexist with this lymphopenia [11]. Numerous independent studies revealed that the amount of NK cells in the bloodstream is also influenced by the SARS-CoV-2 infection [12,13] without differences in the location of NK cell subsets. This decrease in circulating NK cells appears to be related to the illness's severity and the acute stage [14,15]. NK cell counts gradually decrease in patients with a fatal course of the illness following the onset of symptoms, contrary to what has been demonstrated for T and NK cell numbers, which are increased in the latter stages of the illness [12,13,16]. According to recent studies, the quantity of NK cells in hospitalized patients and the rate of viral load reduction are directly related. Circulating NK cell counts may be used as a prognostic clinical parameter to predict the course of COVID-19. Patients with "normal" (>40 cells/L) NK cell numbers experience a faster decline in viral load than patients with "low" (40 cells/L) NK cell numbers, regardless of the clinical status. It is tempting to assume that the SARS-CoV-2 vaccination would be successful in restoring NK cells and their function, given the decreased frequency of NK cells seen in COVID-19 patients. Clinical studies have demonstrated that the BNT162b2 mRNA vaccine boosts cellular and humoral immunity, including the expansion of NK cells [17,18].

In this study, we tracked the development of circulating NK cells after Comirnaty (Pfizer Australia Pty Ltd) vaccination, concentrating on the activation of NK cells, given their relevant role in infection control.

2. Materials and Methods

2.1 Study Population

Twenty healthy volunteers were enlisted for the study. The exclusion criteria were the presence of adverse effects following the vaccination, a history of smoking, and heterologous immunization. The inclusion criteria were the absence of prior COVID-19 infection, the absence of adverse effects, and the absence of comorbidities. Following approval by the Area Vasta Emilia Centro della Regione Emilia-Romagna (CE-AVEC) ethics committee (protocol # 122/2021/Oss/AOUFe), samples were obtained with informed consent. Table 1 lists the demographic characteristics.

2.2 Human PBMCs Isolation

Peripheral blood mononuclear cells (PBMCs) collected 9 months after the second vaccination and 1 month after the booster dose with mRNA vaccines were isolated from the whole blood of healthy donors by centrifugation using Lymphocyte separation medium (LSM; Corning; Merck Life Science S.r.l., Milan, Italy).

Table 1. Demographic and clinical characteristics.

Female, n (%)	50 (10)
Age (years), mean (SD)	36 (10)
Smoker, n (%)	0 (0)
Comorbidities n (%)	0 (0)
mRNA vaccine*	20 (100%)
Blood sample after second immunization (months $\pm$ SD)	9.0 $\pm$ 0.15
Blood sample after booster dose (months $\pm$ SD)	1.0 $\pm$ 0.12

*All the subjects received three doses of Comirnaty (Pfizer Australia Pty Ltd.).

2.3 Antigen Presenting Cell Preparation and Antigen Loading

LSM (Corning; Merck Life Science S.r.l., Milan, Italy) was used to separate peripheral blood mononuclear cells (PBMCs) from whole blood of healthy donors 9 months after the second vaccination and 1 month after the booster dose with mRNA vaccines. Cells were plated at a density of 1×10^6 cells/mL in a T-25 flask and allowed to adhere for 2 h at 37 °C. Nonadherent cells were removed and used to obtain NK cells.

For five days, adherent cells were grown in specific media (CellGro DC, CellGenix; Freiburg, Germany) with 1000 IU/mL of GM-CSF (granulocyte-macrophage colony-stimulating factor) and 50 ng/mL of IL-4 (interleukin-4) (R&D System). Three spike protein antigens (Wuhan, Alpha B.1.1.7, Delta B.1.617.2, and Omicron B1.1.529 full-length spike protein, BioServ, Flemington, NJ, USA) were added to the antigen-presenting cells on day 4 at a concentration of 40 µg/mL for 24 h. Tumor necrosis factor-alpha (20 ng/mL), interleukin-1 (10 ng/mL), and interferon-gamma (1000 IU/mL) were used to stimulate the final antigen following cell maturation for two days [19].

2.4 NK Cell Purification and Stimulation

According to product instructions, NK cells were extracted from peripheral blood samples using the positive magnetic cell separation technique (Miltenyi Biotech, Gladbach, Germany). According to flow cytometry results using CD56-PerCp-Cy5.5 and CD16-FITC moAbs from e-Bioscience in Frankfurt, DE, the NK cell concentration was >90% (data not shown). Autologous pure NK cells were added at a rate of 1.5×10^6/well for 4 h to allow for NK-cell stimulation after plating 1.5×10^5/well spike-loaded antigen-presenting cells into a 12-well plate. As a positive control, NK cells were treated with 25 ng/mL PMA (Sigma, St. Louis, MO, USA) and 1 µM ionomycin (Sigma, St. Louis, MO, USA).

2.5 Granzime ELISpot Assay

A total of 10^6 stimulated NK cells were placed into microplates in a humidified 37 °C CO_2 incubator for 24 h. Granzime-B release was quantified by the Human Granzime B ELIspot assay (R&D Systems, Milan, Italy).

2.6 Flow Cytometry Analysis

Autologous pure NK cells were stimulated with spike-loaded antigen-presenting cells, as reported in Section 2.4. As a positive control, NK cells were treated with 25 ng/mL PMA (Sigma, St. Louis, MO, USA) and 1 µM ionomycin (Sigma, St. Louis, MO, USA). NK cells were stained with the following antibodies (Biolegends): APC mouse anti-human CD3, PerCP-vio700 mouse anti-human CD56, FITC mouse anti-human CD16, PE mouse anti-human NKG2A, NKG2D, NKG2C, CD127, and CD57. The samples were incubated for 30 minutes with the moAbs or anti-isotype controls (Exbio, Praha, CZ) in ice, washed, and analyzed with FACS Aria flow cytometer and FlowJo software (Becton Dickinson, San Jose, CA, USA), acquiring 10,000 events. Lymphocytes were identified according to forward/side scatter profile, and NK cells ($CD3^-/CD56^+$) were defined and gated within the lymphocyte gate (**Supplementary Fig. 1b**). Cell viability was assessed by propidium iodide staining. CD107a degranulation assay was performed after 1 h of incubation at 37 °C and 3 h of treatment with Golgi Stop solution (Becton Dickinson, San Jose, CA, USA).

2.7 Anti-Spike RBD IgG Quantification

Plasma samples collected from vaccinated control subjects were evaluated by commercial ELISA assay for anti-RBD (receptor binding domain) IgG levels (ThermoFisher Scientific, Milan, Italy), following assay protocols. Anti-spike RBD IgG was considered specific for SARS-CoV-2 spike protein when a ratio >1.3 compared to the calibrator was obtained.

2.8 Neutralization Rate Evaluation

In vitro testing was done to determine the anti-SARS-CoV-2 infection-specific anti-spike antibodies' neutralizing power [20]. The SARS-CoV-2 inoculum was donated by Prof. Caruso of the University of Brescia in Italy and was isolated from a nasopharyngeal swab taken from a patient with COVID-19 (a Caucasian man of Italian descent, genome sequences available at GenBank (SARS-CoV-2-UNIBS-AP66: ERR4145453). This SARS-CoV-2 isolate belonged to the B1 clade, which also comprises the majority of Italians and sequences from other European and American nations. Plaque assay was used to measure the virus titer in Vero E6 cells, as previously mentioned [12]. Patient plasma samples were used to cultivate Calu-3/SARS-CoV-2-infected cells. The viral load was determined by real-time PCR for the SARS-CoV-2 genome performed on RNA extracted from cell supernatants, compared to infected controls. The neutralization was reported as the decreased viral load percentage.

2.9 Viral RNA Detection

RNA extraction was performed by using the MagMAX Viral/Pathigen Nucleic Acid Isolation kit (ThermoFisher, Italy) according to the manufacturer's instructions, 48 h post-infection (hpi) [21]. SARS-CoV-2 titration was performed by RealTime-PCR with the TaqMan 2019nCoV assay kit v1 (ThermoFisher, Italy).

2.10 Statistical Analysis

Statistical analysis was performed by a parametric approach for the normal distribution, as assessed using the Kolmogorov–Smirnov test. Fisher's exact test was used to compare percentage frequencies, and Student's *t*-test was used to compare variables. Spearman's correlation test was used to evaluate linear regression. A value of $p < 0.05$ was accepted as statistically significant. The statistical analysis was performed by GraphPad software version 9 (Dotmatics, Boston, MA, USA).

3. Results

3.1 Spike-Binding and Neutralizing Antibody Levels

The sera of healthy donors showed significant spike-binding and neutralizing antibody variability following a second immunization (Fig. 1a,b). We observed an increase in the spike-binding and neutralizing antibody levels 1 month after the booster dose (Fig. 1a,b) (Student's *t*-test, Fisher's exact test, $p = 0.001$ respectively), demonstrating the success of the booster dosage in enhancing the humoral response towards SARS-CoV-2.

3.2 NK Cell Activation and Immunophenotype

We examined the immunological profile of NK cells nine months after the second vaccination and 1 month after the booster dose since the innate immune response is an important part of the protective immune response to infections [22,23]. Between 9 months after the second vaccination and 1 month after the booster dose, we detected no variation in the frequency of $CD3^-CD16^+CD56^{dim}$ and $CD3^-CD16^-CD56^{bright}$ cells (data not shown). Fig. 2a shows the enrichment of $CD16^+CD56^{dim}$ NK cells expressing NKG2A, NKG2C, and NKG2D 1 month after the booster dose (Fig. 2a–c) as compared to 9 months after the second vaccination ($p = 0.001$; Fisher's exact test). At 1 month after the booster dose, CD127, a differentiation marker, was more commonly seen in $CD16^-CD56^{bright}$ NK cells than at nine months following the second immunization (Fig. 2d) ($p = 0.001$; Fisher's exact test). There was no evidence of $CD127^+$ expression in $CD16^+CD56^{dim}$ NK cells. Compared to the group that received the booster dose nine months after the first vaccine, highly mature $CD57^+$ NK cells were especially abundant among $CD16^+CD56^{dim}$ NK cells (Fig. 2e) ($p = 0.001$; Fisher's exact test). There

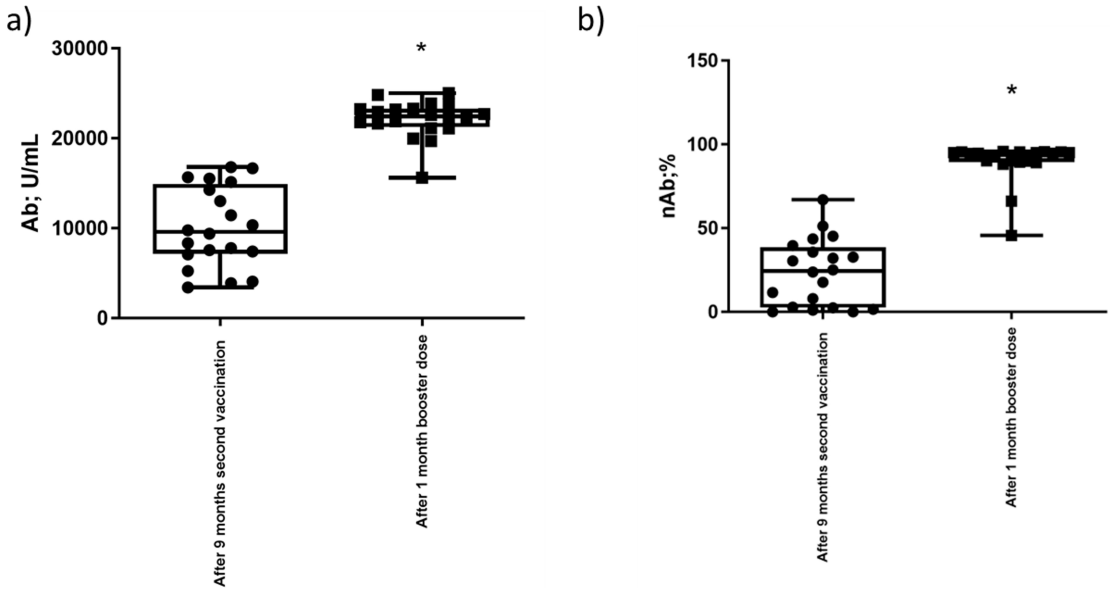

Fig. 1. Anti-Spike IgG evaluation. (a) Anti-spike SARS-CoV-2 RBD IgG (Ab) plasma levels in 20 healthy individuals 9 months after the second vaccination and 1 month after the booster dose. The results are reported as the mean ± standard deviation. *p values evaluated by the Students t-test. (b) Percentage of inhibition of SARS-CoV-2 infection of Calu3 in co-culture with plasma samples of 20 healthy individuals 9 months after the second vaccination and 1 month after the booster dose. The results are reported as the mean ± standard deviation. *p values evaluated by Fisher's exact test.

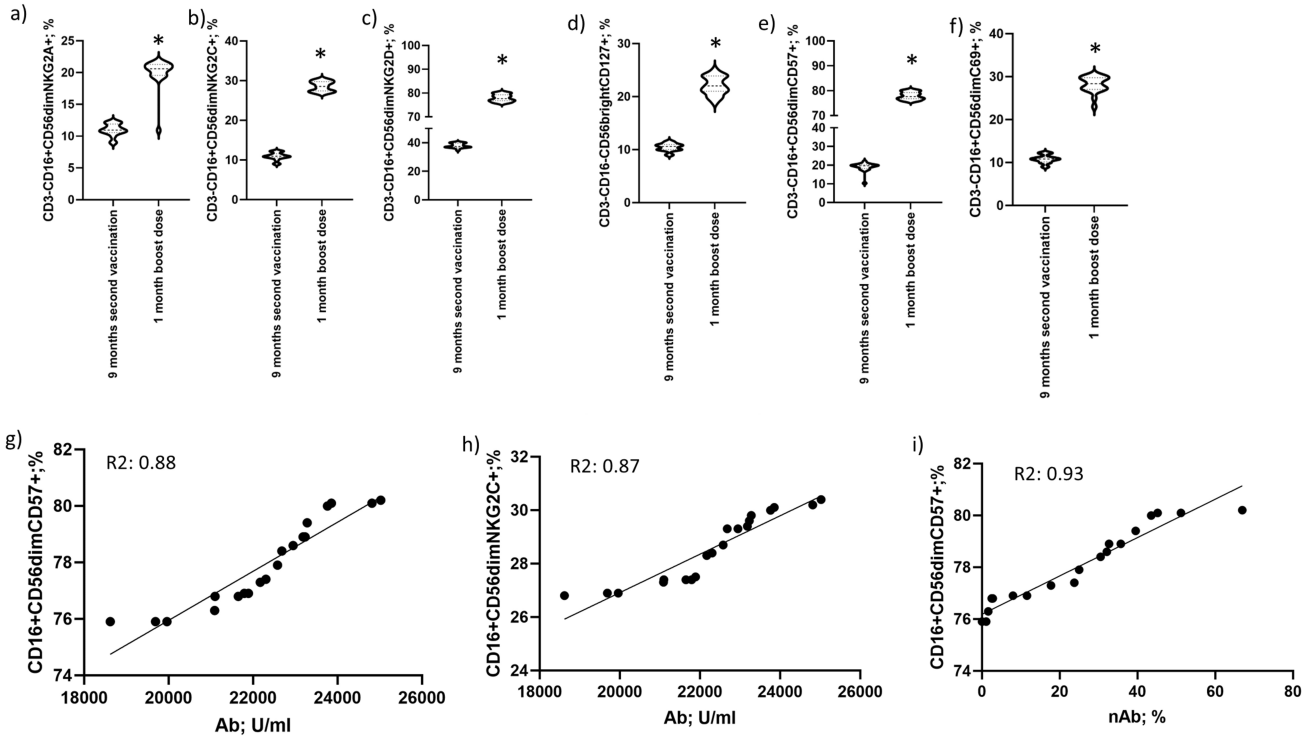

Fig. 2. NK cell immunophenotype. Differential profile of $CD56^{dim}$ and $CD56^{bright}$ NK cells 9 months after the second vaccination and 1 month after boost dose. The frequency of $CD56^{dim}$ NK cells expressing (a) NKG2A, (b) NKG2C, (c) NKG2D, (e) CD57, and (f) CD69, and of $CD56^{bright}$ NK cells expressing (d) CD127 in the peripheral blood of vaccinated subjects was assessed by flow cytometry. The results are reported as the mean ± standard deviation. * significant p values evaluated by Fisher's exact test. The expansion of $CD56^{dim}$ $NKG2C^{+}$ or $CD57^{+}$ NK cells in vaccinated subjects, 1 month after boost dose, according to SARS-CoV-2 seropositivity. Correlation between (g) $CD56^{dim}CD57^{+}$, (h) $CD56^{dim}NKG2C^{+}$ cells and SARS-CoV-2 serology. (i) Correlation between $CD56^{dim}CD57^{+}$ cells and neutralizing SARS-CoV-2 serology. r^2 values were evaluated by Spearman's correlation test.

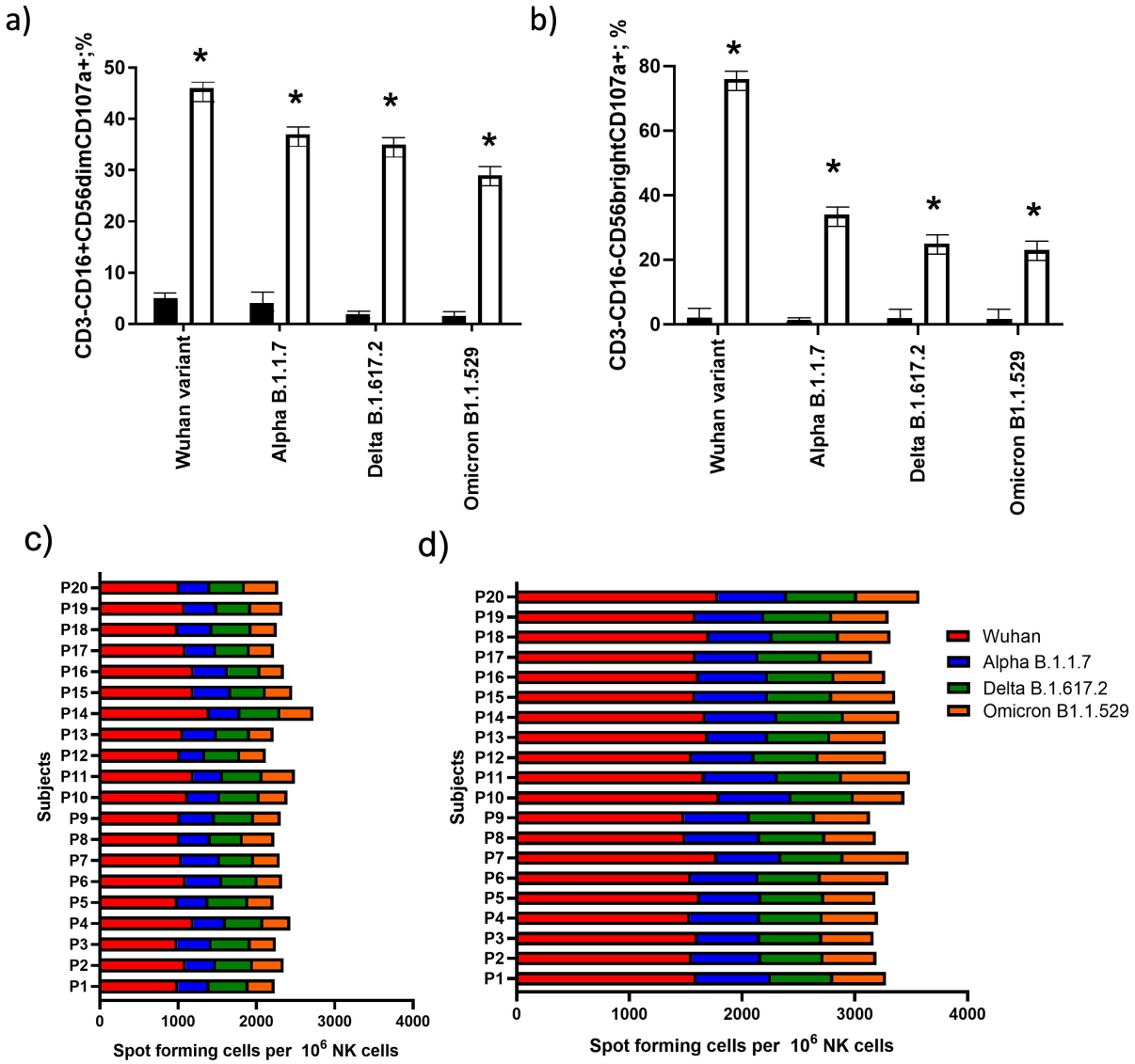

Fig. 3. NK cell activation and subset evaluation. The number of (a) $CD56^{dim}$ and (b) $CD56^{bright}$ NK cells expressing CD107a stimulated with Wuhan, Alpha B.1.1.7, Delta B.1.617.2, Omicron B1.1.529 variants. Black histogram: 9 months after the second vaccination; white histogram: 1 month after the booster dose. The results are reported as the mean ± standard deviation. *p values evaluated by Fisher's exact test. Spot forming cell per 10^6 NK cells secreting Granzime-B after stimulation with Wuhan, Alpha B.1.1.7, Delta B.1.617.2, Omicron B1.1.529 variants. (c) 9 months after the second vaccination. (d) 1 month after the booster dose.

was no evidence of CD57 expression in $CD16^{-}CD56^{bright}$ NK cells. Additionally, in the month after the booster dose (Fig. 2d) ($p = 0.001$; Fisher's exact test), the percentage of $CD16^{+}CD56^{dim}CD69^{+}$ NK cells increased.

The results revealed considerable differences in NK surface markers expression 1 month after the booster dose, including a high level of NKG2A/C/D^{+} and $CD57^{+}$ in $CD56^{dim}CD16^{+}$ NK subsets and an increase of $CD16^{-}CD56^{bright}CD127^{+}$ NK cells.

3.3 Expansion of $NKG2C^{+}$ and $CD57^{+}$ NK Cells, and SARS-CoV-2 Levels of Seropositivity

Since there is evidence that at least some NK-cell subsets, such as the rise in $NKG2C^{+}CD57^{+}$ NK cells after viral infection, are involved in the adaptive immune response to particular antigens [19,21], we looked at the relationship between $CD16^{+}CD56^{dim}NKG2C^{+}$ or $CD57^{+}$ NK cell numbers and SARS-CoV-2 seropositivity. In the 1-month post-booster dose group, the percentages of $CD16^{+}CD56^{dim}NKG2C^{+}$ or $CD16^{+}CD56^{dim}CD57^{+}$ cells (Fig. 2h,i) were significantly linked with the SARS-CoV-2 serology (r^2: 0.88, 0.87, respectively; Spearman's correlation test).

3.4 Activation Markers in NK Subsets

We determined how frequently NK cells with $CD16^{-}CD56^{bright}$ and $CD16^{+}CD56^{dim}$ phenotypes released Granzime-B and degranulated (expressed CD107a) after being stimulated by Spike. When compared to 9 months after the second vaccination, the group that received the booster dose showed enrichment of $CD16^{+}CD56^{dim}CD107a^{+}$ cells, with the Wuhan variant reaching a frequency of 46%, followed by the Alpha B.1.1.7 (37%), Delta B.1.617.2 (35%), and Omicron B1.1.529 (29%; Fig. 3a). Following 1 month in the booster group, $CD16^{-}CD56^{bright}CD107a^{+}$ cells were most frequently observed (76%), and they were less frequently observed (34%), (25%) and (23%), respectively, 1 month after challenges with the variants Alpha B.1.1.7, Delta B.1.617.2, and Omicron B1.1.529 in Fig. 3b. In a similar manner, the percentage of Granzime-B^{+} NK cells increased after the booster dose when challenged with the Wuhan, Alpha B.1.1.7, Delta B.1.617.2, and Omicron B1.1.529 variants (Fig. 3b; $p = 0.001$, Fisher's exact test), with the Wuhan variant inducing an increased percentage of Granzime-B^{+} NK cells in both the 9 months following the second vaccination and the booster dose time points (Fig. 3b; p0.001).

The 1-month post-boost dose group showed enrichment of both $CD56^{bright}CD16^{-}CD107a^{+}$ and $CD56^{dim}CD16^{+}$Granzime-B^{+} NK cells.

4. Discussion

The elimination of viruses such as SARS-CoV-2 is known to be significantly assisted by NK cells [24]. The production of activated NK cells (i.e., expressing CD69 and CD107a) is essential for the success of vaccines. In order to further evaluate NK cell activity, we evaluated $CD3^{-}CD16^{+}CD56^{dim}$ and $CD16^{-}CD56^{bright}$ blood cells, as well as the expression of surface receptors and activation markers in these cells [25].

One month following the booster dose, we identified a unique NK-cell profile that was enriched for maturing NK "memory" $CD56^{dim}CD57^{+}$ cells [26], immature $CD127^{+}$ expression [27,28], and $CD107a^{+}$ and/or Granzime-B secreting cells. In addition, the patients had $CD56^{dim}$ cells that expressed $NKG2A^{+}NKG2C^{+}NKG2D^{+}$. These findings showed that after receiving the booster dose for a month, the participants had an unusually activated NK-cell profile, most likely as a result of long-term exposure to SARS-CoV-2.

After one month of a booster dose, CD127 (IL-7 receptor chain) expression on $CD56^{bright}$ NK cells increased. This was a recognizable change to the NK-cell surface marker. The prospect of dynamic replacement in NK cells is increased by the hypothesis that $CD127^{+}$ NK cells are derived from the thymus. A subgroup of NK cells that express CD127 in mice lymph nodes is also thought to originate from the thymus [27]. Studies have documented the accumulation of immature $CD127^{+}$ NK cells in mice with developing tumors or those with persistent viral infections [28]. More studies are required to evaluate whether $CD56^{bright}$ $CD127^{+}$ NK cells in the peripheral blood of humans may be cells migrating from the thymus and homing to other sites.

NKG2D is an activating receptor essential for the antiviral and anticancer functions of NK cells. NK-cell $CD56^{dim}$ subsets exhibited a significant increase in NKG2D expression one month after the boost dose. This finding may be related to SARS-CoV-2 resistance. SARS-CoV-2 S protein peptides that interact with the NKG2D receptor have been found through the use of in silico analysis. Cov1 and Cov2 were able to bind to NKG2D receptors and NK cells, respectively. These peptides induced NK cytotoxicity against lung cancer cells and promoted interferon-gamma secretion by NK cells by phosphorylating Vav1, a downstream-signaling protein of NK activation genes [29].

Individuals showed increased Granzime-B synthesis one month after the boost dose, as well as increased CD69 expression, a hallmark of cell activation [30], in $CD56^{dim}$ NK cells, and CD107a degranulation [31] in both $CD56^{dim}$ and $CD56^{bright}$ NK cells. Despite the fact that the $CD56^{bright}$ subset is normally thought to be more secretory, the proportion of $CD107a^{+}$ cells significantly increased. It is important to note that the four different SARS-CoV-2 genotypes that were evaluated increase the production of CD107a and Granzime-B. These results suggest that different NK-cell subsets may activate to different degrees in the peripheral blood following a booster dosage and subsequent interaction with SARS-CoV-2 variants.

The purpose of NK cells that simultaneously express numerous receptors is unknown; however, it is most likely involved in the activation or inhibition of NK-cell subsets. It has been noted that SARS-CoV-2 causes an increase in HLA-E expression [32], which causes NK cells to perform less effectively for protection against the virus. It's intriguing to observe that 1 month following the booster dose, CD57 and NKG2C were substantially expressed on $CD56^{dim}$ NK cells, and there was a clear correlation between this expression and IgG titers for SARS-CoV-2. We hypothesize that repeated anti-SARS-CoV-2 vaccinations are what led to the establishment of memory-like NK cells following the booster dosage, which has not yet been described.

5. Conclusions

Activating profiles with enhanced $NKG2D^{+}$ expression and an increased percentage of $CD107a^{+}$ and Granzime B-expressing cells within both the $CD56^{dim}$ and $CD56^{bright}$ NK-cell subsets were detected in patients 1 month after the first dosage. The presence of memory-like $CD56^{dim}CD57^{+}NKG2C^{+}$ cells, an activating phenotype, as well as the increase in CD127 expression in NK $CD56^{bright}$ cells, confirm a high frequency of immature and thymic-derived cells. More research is needed to determine

how the immune system reaction to SARS-CoV-2 is influenced by the expression of activating/inhibitory receptors on cytotoxic $CD56^{bright}$ and $CD56^{dim}$ NK cells and regulatory $CD56^{bright}$ NK cells. These findings are encouraging since the majority of vaccine-induced NK cell responses are still able to distinguish between various SARS-CoV-2 variants. Nevertheless, it is crucial to monitor any variations that can lead to a potential decline in NK cell responses.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

These should be presented as follows: RR, MC, GZ designed the research study. LM collected data and patients. SR, GS, SB, FC, VG performed the research. VG, DB, AP analyzed the data. GZ, AP, RR, MC wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of the Area Vasta Emilia Centro della Regione Emilia-Romagna (CE-AVEC) with the number 122/2021/Oss/AOUFe. Informed consent was obtained from all subjects involved in the study.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2807156.

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