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RESEARCH ARTICLE

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Long-term dynamics of natural killer cells in response to SARS-CoV-2 vaccination: Persistently enhanced activity postvaccination

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Abstract

Natural Killer (NK) cells play a significant role in the early defense against virus infections and cancer. Recent studies have demonstrated the involvement of NK cells in both the induction and effector phases of vaccine-induced immunity in various contexts. However, their role in shaping immune responses following SARS-CoV-2 vaccination remains poorly understood. To address this matter, we conducted a comprehensive analysis of NK cell phenotype and function in SARS-CoV-2 unexposed individuals who received the BNT162b2 vaccine. We employed a longitudinal study design and utilized a panel of 53 15-mer overlapping peptides covering the receptor binding domain (RBD) of the SARS-CoV-2 Spike protein to assess NK cell function at 0 and 20 days following the first vaccine, and 30 and 240 days following booster. Additionally, we evaluated the levels of total IgG anti-Spike antibodies and their potential neutralizing ability. Our findings revealed an increased NK cell activity upon re-exposure to RBD when combined with IL12 and IL18 several months after booster. Concurrently, we observed that the frequencies of

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NKG2A + NK cells declined over the course of the follow-up period, while NKG2C increased only in CMV positive subjects. The finding that NK cell functions are inducible 9 months after vaccination upon re-exposure to RBD and cytokines, sheds light on the role of NK cells in contributing to SARS-CoV-2 vaccine-induced immune protection and pave the way to further studies in the field.

KEYWORDS

CD107a, natural killer cells, NKG2A, NKG2C, SARS-CoV-2, vaccine

1 | INTRODUCTION

Natural Killer (NK) cells play a crucial role in recognizing and eliminating virus-infected and tumor cells through activating receptors that trigger a functional response. This response is regulated by inhibitory receptors that recognize HLA-I molecules. While NK cells have traditionally been considered as part of innate immunity, recent studies have challenged this notion by demonstrating their ability to recognize previously encountered pathogens.[1–4] The discovery of adaptive/memory NK cells and their enhanced responses upon reencountering pathogens opens new possibilities for controlling infectious diseases and raises the question of whether pathogen-specific NK cell responses can be induced by vaccination. Such "memory" or "adaptive" NK cells (mNK), have been identified in association with CMV infection [5–7] and are characterized by the lack of FcεR1 adaptor protein and by the expansion of NKG2C + NK cell population.[8,9] NKG2C and NKG2A are two receptors that form a heterodimer with CD94. They share the same ligand, the nonclassical major histocompatibility complex class I molecule HLA-E, but their role is different, NKG2C being an activating receptor and NKG2A having inhibitory function. NKG2A has an immunoreceptor tyrosine-based inhibitory motif (ITIM) on its cytoplasmic tail, which transmits inhibitory signals, while NKG2C interacts with the adaptor protein DAP12, that has an immunoreceptor tyrosine-based activation motif (ITAM) for NK cell activation.[10] Since NKG2A has a higher affinity for HLA-E, inhibitory signals prevail over activation. The interaction of NKG2C and HLA-E loaded with CMV UL-40 peptides induces a peptide-specific activation and expansion of mNK cells.[11,12] In the setting of COVID-19 infection, the deletion of NKG2C receptor has been associated with the development of severe COVID-19.[13] Moreover, we and others have observed an expansion of memory NK cells in individuals with severe COVID-19 infection, particularly in those who succumbed.[14,15] Of note, it has recently been shown that a SARS-CoV-2 peptide from the nonstructural protein 13 (nsp13) prevents interaction between HLA-E and the inhibitory receptor NKG2A, abrogating NKG2A-mediated inhibition and unleashing NK cell activity.[16]

The ability of NK cells to respond to peptides makes them suitable for pathogen-specific immunological control after vaccination and increasing data support their contribution to both the induction and the effector phases in response to different vaccines.[17–20] Recently, vaccine induced memory NK cells have been found in the context of Flu,[21] Ebola,[22,23] and hepatitis B [2] vaccines.

Similar long-lived memory-like NK cells have been generated by cytokine activation[24]; however, it is not known if this cytokine induced memory cells are comparable to those emerging from exposure to CMV. Cytokine-Induced Memory-Like (CIML) NK cells, were initially identified in vitro after multiple cytokine stimulation (IL12, IL15 and IL18). Enhanced IFNγ secretion and cytotoxicity have been described in these cells after restimulation with the previously mentioned cytokines or IL12 + IL15 or K562 target cell.[25,26]

Interestingly, epigenetic modifications have been observed in CIML NK cells, and similar modifications have been reported in the expanded NKG2C$^+$ mNK cell subset in human CMV-infected subjects,[8] suggesting that signaling through NKG2C mediated by CMV peptide-loaded HLA-E, with or without additional cytokine stimuli, also results in sustained functional modification of NK cells.

In this study, we investigated the relevance of classical and adaptive/memory NK cells in SARS-CoV-2 vaccinated individuals by characterizing NK cell phenotype and function at baseline and at different time points after vaccination. Our findings revealed a significant increase in NK cell responses following stimulation of peripheral blood mononuclear cells (PBMCs) with SARS-CoV-2 RBD overlapping peptides. We also observed that NKG2C positive NK cells exhibited superior degranulation activity and IFNγ secretion compared with NKG2C negative NK cells at all time points.

2 | SUBJECTS

Peripheral blood mononuclear cells were collected from 23 (15 females/8 males; median age 36 years), BNT162b2 mRNA vaccinated individuals at four timepoints: before vaccination (T0), 20 days after first dose (T1), and 1 (T2) and 8 (T3) months after booster.

More specifically, blood was collected just before the first dose of vaccine at T0, 20 days after T0 (T1), 1 month after T1 (T2), and 7 months after T2 (T3). All subjects were tested for CMV IgG antibodies: 15 were IgG positive and the remaining 8 were IgG negative.

Subjects were health care workers unexposed to SARS-CoV-2, based on absence of anti-N antibodies for the entire duration of the follow up. Demographic information is shown in Table S1. All subjects signed an informed consent. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Board and Ethical

Committee of Fondazione IRCCS Policlinico San Matteo (document number 20210006653).

3 | METHODS

3.1 | Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated by Lympholyte (Cedarlane, Burlington, Canada) density gradient (1.077 g/ml) centrifugation according to the manufacturer's instructions. Cells were frozen in 90% fetal bovine serum (FBS) 10% dimethylsulphoxide (DMSO) and stored in liquid nitrogen. Briefly, whole blood was diluted with an equal volume of phosphate-buffered saline (PBS) and layered on the gradient. After centrifugation at 500 × g for 30 min at room temperature PBMC were resuspended in PBS-EDTA and centrifuged at 400 × g for 10 min. Pellets were resuspended in PBS containing 2% FBS and washed by centrifugation at 250 × g for 10' at room temperature. Cell numbers were determined by light microscopy count in a Burker chamber. Nonviable cells were identified by staining with trypan blue.

Cryopreserved PBMC were thawed, washed, and rested for 30 min in complete RPMI-1640 medium supplemented with 10% FBS, 2Mm L-glutamine and antibiotic antimycotic solution (100 U/ml penicillin, 0.1 μg/ml streptomycin, Sigma-Aldrich, St. Louis, MO, USA).

3.2 | Purification of natural killer cells

NK cells were isolated by means of negative selection using EasySep Human NK Cell Isolation Kit (StemCell Technologies, BC, Canada) following the manufacturer's instructions. NK cell purity was >90%.

3.3 | NK and T cell phenotype

5×10^5 PBMC were used for immunophenotypic analysis using a 12-color FACS Celesta flow cytometer (BD Biosciences, CA, USA). LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher Scientific Walthman, USA) was used to determine cell viability. The monoclonal antibody panel used to characterize the main lymphocytic subsets is available in Table S2. Staining of FcεRIγ adaptor protein was performed using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, CA, USA) according to the manufacturer's instructions.

3.4 | Peptide pools and antigens

Fifty-three 15-mer peptide pools overlapping by 11 aminoacid residues, covering the Receptor-Binding Domain (RBD) of the ancestral Wuhan sequence (GenBank: MN_908947), were synthesized by ChinaPeptides (Shanghai, China). Purity of peptides was ≥ 90%. Each lyophilized peptide was resuspended in 50% DMSO at a concentration of 10 mg/ml, and pooled. The final mega pool was used at the concentration of 3 μg/ml. Negative control was a matched DMSO concentration.

3.4.1 | NK cell function

Functional analysis was performed with thawed PBMC after 2 h resting in complete medium.

10^6 rested PBMC for each time point were stimulated with and without IL12 (10 ng/ml, R&D System, MN, USA), IL18 (100 ng/ml, R&D System), in the presence or absence of SARS-CoV-2 RBD 15-mer overlapping peptides (OP) (3 μg/ml, China Peptides), as described above for 18 h, in complete RPMI medium. CD107a BV786 (BD Biosciences) was added in culture and brefeldin A (GolgiPlug, BD Biosciences) was also added during the last 12 h. Cells were then membrane stained with the following antibodies: anti-CD3, -CD56, -NKG2C, -CD8, -CD57 (details are listed in Table S1). After fixation and permeabilization (Fixation/Permeabilization Solution Kit, BD Biosciences), cells were stained with anti-IFNγ (Table S1). Analysis was performed by FACS Celesta (BD Biosciences).

3.4.2 | Ex vivo enzyme-linked immunospot (ELISpot) assay

Antigen specific T cell responses were evaluated by IFNγ detection after overlapping Spike 15-mer peptide stimulation in an ELISpot assay according to the manufacturer's instructions (Mabtech). After 2 h resting in complete medium, thawed PBMC were seeded at 2×10^5 cells/well in 96-well plates pre-coated with anti- IFNγ (15 μg/ml; clone 1-D1K; Mabtech, Stockholm, Sweden). Test wells were supplemented with the above peptides (1 μg/ml, PepTivator® SARS-CoV-2 Prot_S Complete, Miltenyi Biotec, Germany). Negative control wells lacked peptides, and anti-CD3 mAb (1:1000; clone CD3-2, Mabtech) was used as positive control. Spots were counted using an automated ELISpot Reader System (Autoimmun Diagnostika GmbH, Strasburg, Germany). Results were given as IFNγ spot forming units (SFU)/10^6 PBMC, after subtracting spots from negative control. The positive cut-off was set at 10 IFNγ SFU/10^6 PBMC.

3.4.3 | Anti-SARS-CoV-2 spike and anti-nucleocapsid serum antibody detection

SARS-CoV-2-specific antibodies in serum samples were analyzed using Liason SARS-CoV-2 IgG chemiluminescent immunoassay (DiaSorin, Saluggia, Italy) for the quantitative detection of anti-S1 and anti-S2 IgG antibodies, according to the manufacturer's instructions. Results were given as AU/Ml and a cut-off of 13 AU/ml was considered for the definition of positive samples.

Anti-Nucleocapsid IgG antibody were measured by a semi-quantitative CMIA assay with Abbott Alinity i SARS-CoV-2 anti-nucleocapsid protein IgG assay according to the manufacturer's instructions. Results are reported as index values, and a positive cutoff of 1.40 was used for the definition of positive samples, as instructed by the manufacturer.

3.5 | Surrogate neutralizing antibody kinetics

The presence of neutralizing/blocking antibodies in serum samples was investigated with the SARS-CoV-2 Neutralizing Antibodies Detection Kit (AdipoGen Life Sciences, Liestal, Switzerland) according to manufacturer's instructions. Neutralizing activity was measured by competitive inhibition between a recombinant SARS-CoV-2 RBD protein and a recombinant human ACE2 receptor (hACE2) in the presence of test serum. The presence of neutralizing antibodies against SARS-CoV-2 in the serum was measured by calculating the percent inhibition of each sample using the following formula: = [1-(OD of the sample)/(OD of Negative Control)] x 100. Samples were classified as either "positive" (inhibition ≥ 20%) or "negative" (inhibition < 20%) as suggested by the manufacturer.

3.6 | Cell lines

Permissive Vero-E6 cells (VERO C1008 (Vero 76, cloneE6, Vero E6); ATCC® CRL-1586™) were cultured in MEM (Gibco) supplemented with 10% heat-inactivated FBS and 100 µg/ml Penicillin, Streptomycin, Glutamine (Euroclone SpA) solution at 37°C in atmosphere of 5% CO_2.

3.7 | Severe acute respiratory syndrome coronavirus 2 strains

SARS-CoV-2 Italian strain PV10734 (D614G, lineage B.1.1) was isolated from infected patients' nasal swabs and stock virus prepared by collecting the supernatant from Vero-E6 cells as previously described.[27] Variant's titer was measured at the 50% tissue culture infectious dose (TCID50) in six replicates using a 96-well flat-bottom tissue-culture microtiter plate as previously described [28]

3.8 | ADCC assay

3×10^5 Vero-E6 cells per well were seeded in a 24 well/plate. The day after, SARS-CoV-2 isolate strain D614G, lineage B.1.1 was added to cells at 100 X TCID50 and incubated at 33° C in 5% CO_2 for 1 h. After inoculum removal, fresh Eagle's MEM (EMEM, Lonza Group Ltd., Basel, Switzerland) supplemented with 1% v/v Penicillin, Streptomycin, Glutamine (Euroclone SpA) and 0.1% v/v Trypsin, was added. The infection was carried out for 48 h.

Cell-surface expression of CD107a was used as a marker for NK cell degranulation. Briefly, 3×10^4 infected and noninfected cell line were seeded and incubated with or without plasma from vaccinated individuals diluted 1:100 for 30 min at room temperature. At the end of incubation, PBMC were added at a 10:1 ratio in the presence of brefeldin A (GolgiPlug, 1 µl/ml, BD Biosciences) and anti-CD107a-BV786 (BD Biosciences) in 96-well flat bottom plates for 4 h at 37° C in a humidified 5% CO_2 atmosphere.

3.9 | HCMV serology

HCMV IgG serology was determined by DRG IgG CMV ELISA kit (DRG International, NJ, USA).

3.10 | Statistical analysis

Statistical analysis was performed using the GraphPad Prism 8.4.3 software (GraphPad, La Jolla, CA, USA). Data were analyzed for their distribution by the D'Agostino & Pearson normality test. Statistical differences between each time point were assessed by repeated measures ANOVA followed by Dunnett's multiple comparison test or mixed effect analysis followed by Tukey's multiple comparison test, as appropriate. Wilcoxon paired *t* test was used to compare paired data within the same timepoint. Correlations between variables were analyzed by Pearson's rank correlation coefficient. Differences were deemed statistically significant when $p \leq 0.05$.

4 | RESULTS

4.1 | Adaptive immune responses to SARS-COV-2 are conserved in vaccinated individuals

Blood was obtained from vaccinated individuals after receiving the BNT162b2 mRNA vaccine. Anti-SARS-CoV-2 Spike specific IgG were tested before vaccination, 20 days after the first dose, 1 month after receiving the second dose and 8 months after the booster. Figure 1A shows the timeline of blood sampling. To determine if subjects were exposed to SARS-CoV-2 we tested sera for anti-N antibodies at all time points and found that none were positive (Figure 1B). Spike-specific IgG were significantly increased at all timepoints when compared with T0. Consistent with existing data in the field,[29–31] Spike specific IgG were significantly decreased at T3 compared with T2, thus confirming the finding of reduced humoral immunity months after vaccination (Figure S1A). Neutralizing antibodies (NAb) activity was undetectable in all subjects at baseline (inhibition <20%). All subjects showed a significant increase in NAb activity at all timepoints, while a NAb decline was evident at T3, in line with total Spike-specific IgG (Figure 1C).

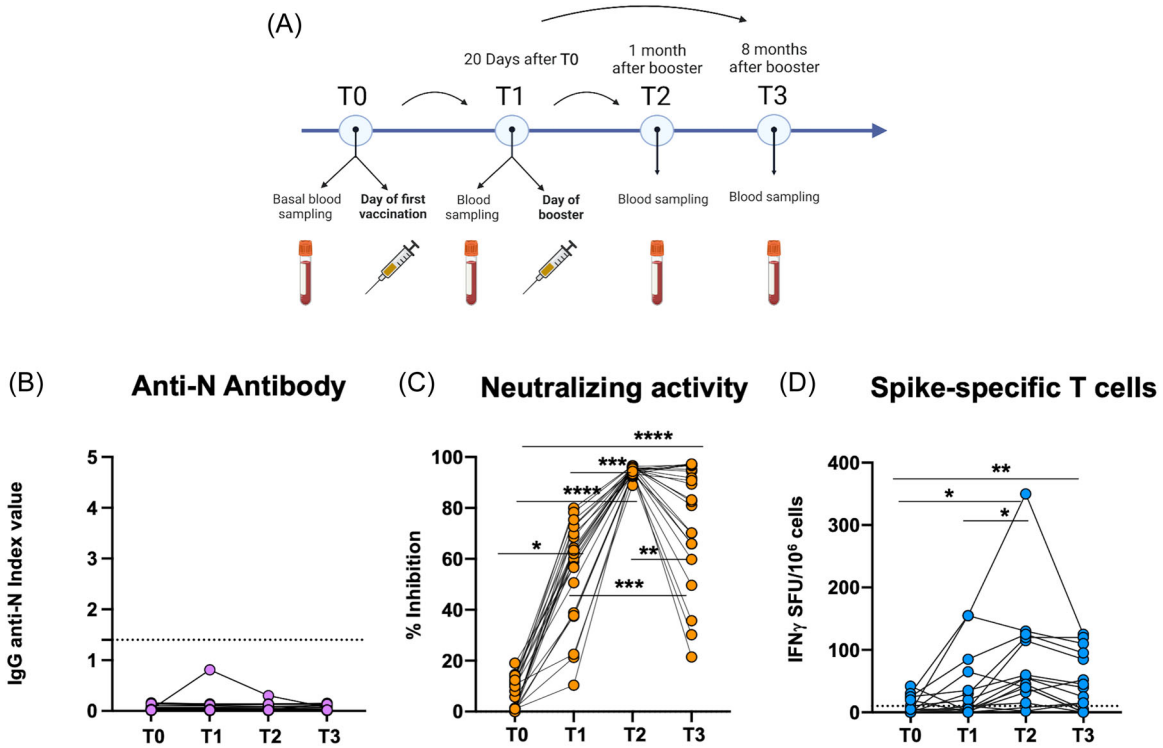

FIGURE 1 Vaccination timeline and adaptive immune responses to BNT162b2 vaccine. (A) The timeline illustrates the different sampling schedule of the study. Each vertical bar represents a sampling time point indicated on the line. Study participants received two doses of the vaccine as shown in the figure. (B) Evaluation of total serum anti-N IgG at T0, T1, T2 and T3; the y-axis represents the antibody index, while the x-axis displays the time points. (C). Neutralizing activity of antibodies specific for the Receptor Binding Domain of SARS-CoV-2 as a function of time. The y-axis reports the percentage of neutralization, while the x-axis shows the timepoints at which the assay was performed. (D). T-cell responses to SARS-CoV-2 Spike Protein. The y-axis indicates the number of spots per 10^6 cells, that is, the frequency of T cells that specifically recognize the Spike protein. The x-axis shows the time points at which T cells responses were measured. The dashed line indicates the cut-off set at 10 SFU/10^6 cells. Statistical comparison between time points was assessed by mixed effect model and Tukey's multiple comparison test *$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Spike specific T-cell responses were assessed by ELISpot assay in 15 subjects after stimulation with Spike peptides at all timepoints. There was a progressive significant increase in IFNγ secretion reaching a peak at T2 and declining, though not significantly so, at T3 (Figure 1D). RBD-specific T-cell responses showed no differences in CD4+ and CD8 + T cells under unstimulated conditions (Figure S1B, C). Cytokine stimulation, instead, augmented IFNγ secretion at all timepoints in CD4+ and at T1, T2, and T3 in CD8 + T cells (Figure S1D, E).

4.2 | Reduced NKG2A/NKG2C ratio after BNT162b2 mRNA vaccination

Frequencies of total NK cells, CD56^{bright}/CD56^{dim}, CD56 + CD57+ FcεRIγ negative adaptive/memory NK cells, CD57 and CD16 NK cells were not significantly different between time points (Figure 2A–F). However, there was a notable decrease in the proportion of NK cells expressing TIM-3 at T3 compared with T0 and T2 (Figure 2G). In addition, the frequency of NK cells expressing the inhibitory CD94/NKG2A heterodimer was significantly reduced at T3 compared with the remaining time points (Figure 3A), though the difference in mean fluorescence intensity (MFI) expression did not reach statistical significance (Friedman test followed by Dunn's multiple comparison test between T3 and T0 $p = 0.057$) (Figure 3B).

While the frequency of the activating CD94/NKG2C heterodimer was not significantly increased (Figure 3C), there was a statistically significant increase in MFI at T3 compared with T2 (Figure 3D).

Interestingly, the NKG2A/NKG2C ratio was reduced at T3 compared with baseline, both as percentage (Figure 3E) and MFI expression (Figure 3F), indicating a potential shift in the balance between these two receptors. Furthermore, when we excluded CMV negative subjects (8/23) from the analysis, we observed a statistically significant increase in the frequency (Figure 3G) and in MFI (Figure 3H) of NKG2C + NK cell at T3 compared with T0. Figure S2 shows representative dot plots of NKG2A+ and NKG2C + NK cells. No significant differences were observed in the other NK cell receptors analyzed in the present study (Figure S3). Representative flow plots showing the gating strategies for the identification of most NK cell receptors analyzed are shown in Figures S4 and S5.

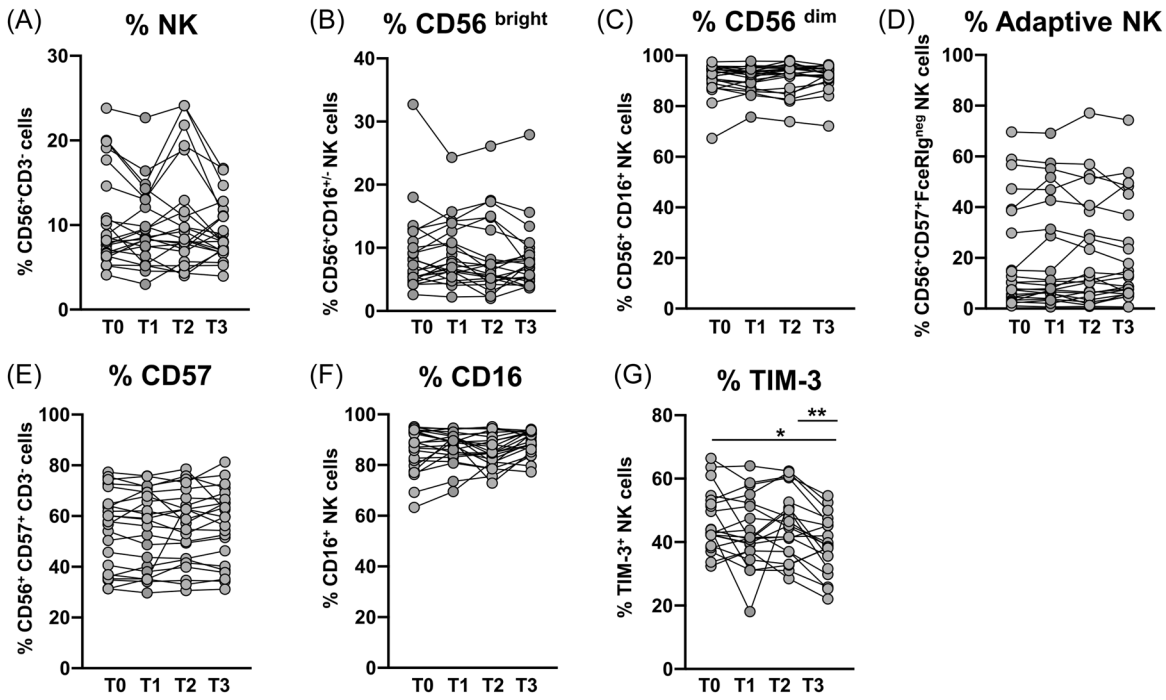

FIGURE 2 NK cell phenotypic changes after BNT162b1 vaccination. Frequencies of (A) total, (B) CD56^{bright}, (C) CD56^{dim}, (D) adaptive, (E) CD57 + , (F) CD16 + , (G) TIM-3 + NK cell subsets. The one-way Friedman with Dunn's multiple comparison test was used to compare data between the 4 timepoints. *$p < 0.05$; ** $p < 0.01$.

Considering the pivotal role played by T cells in coordinating vaccine responses, we also analyzed subpopulations of CD4 and CD8 T cells, including naïve, central memory (CM), effector memory (EM), and effector memory CD45RA positive (EMRA) cells. The frequency of EMRA CD4 + T cells was significantly increased at T2 compared with T1 (Figure 6A), while other T cell subpopulations showed no significant changes over time (Figures S6B–D, 6F–I). Similarly to NK cells, TIM-3 expression was significantly reduced at T3 in CD4+ and CD8 + T cells (Figures S6E, 6J).

4.3 | SARS-CoV-2 receptor binding domain enhances NK cell function 8 months after vaccine booster

Comparison between RBD-stimulated and unstimulated PBMC in the absence of cytokines showed that RBD stimulation significantly increased IFNγ secretion at T1, T2, and T3 (Figure 4A), while the frequency of CD107a-positive NK cells was significantly higher only at T1 and T3 (Figure 4B). Addition of IL12 and IL18 to the system resulted in a significantly increased IFNγ production by NK cells at all time points after RBD stimulation compared with IFNγ secretion without peptides (Figure 4C). Notably, CD107a was also upregulated on NK cells by RBD at all timepoints (Figure 4D). Longitudinal comparison of NK cell function showed a significant increase in IFNγ secretion at T3 compared with T2, but only when IL12 and IL18 were used as a stimulus (Figure 4C). Similarly, cytokine stimulation enhanced CD107a degranulation at T3 compared with T0 and T2 in the presence of RBD (Figure 4D, E). Notably, preliminary experiments performed with purified NK cells did not show any increase in IFNγ secretion and CD107a expression under identical experimental conditions, indicating that other cells are needed to stimulate NK cell function (Figure S7).

Interestingly, there was a positive correlation between the frequencies of IFNγ + NK cells at T1 and IFNγ + CD4+ (Figure 5A, B) and IFNγ + CD8+ (Figure 5C, D) T cells at T1 and T2 in the absence of cytokine stimulation.

Adaptive/memory NK cells are known to exhibit reduced expression of FcεRIγ and to express the NKG2C activating receptor. To assess adaptive NK cell responses, the ability of NKG2C-positive and negative NK cells to respond to RBD stimulation was compared (gating strategy in Figure S8). RBD stimulation significantly increased NK cell degranulation and IFNγ production at all time points in NKG2C-positive cells compared with NKG2C-negative cells in unstimulated PBMC (Figure 6A-B). Similarly, NKG2C positive NK cells showed increased cytotoxic potential after cytokine stimulation compared with NKG2C negative NK cells at T1, T2 and T3 (Figure 6C). However, IL12 + IL18 stimulation increased IFNγ secretion from NKG2C positive cells only at T2 (Figure 6D). Except for IFNγ levels at T3, in the absence of peptides, IFNγ secretion and CD107a degranulation were similar in both populations with and without IL12 + IL18 stimulation (Figure 6E–H).

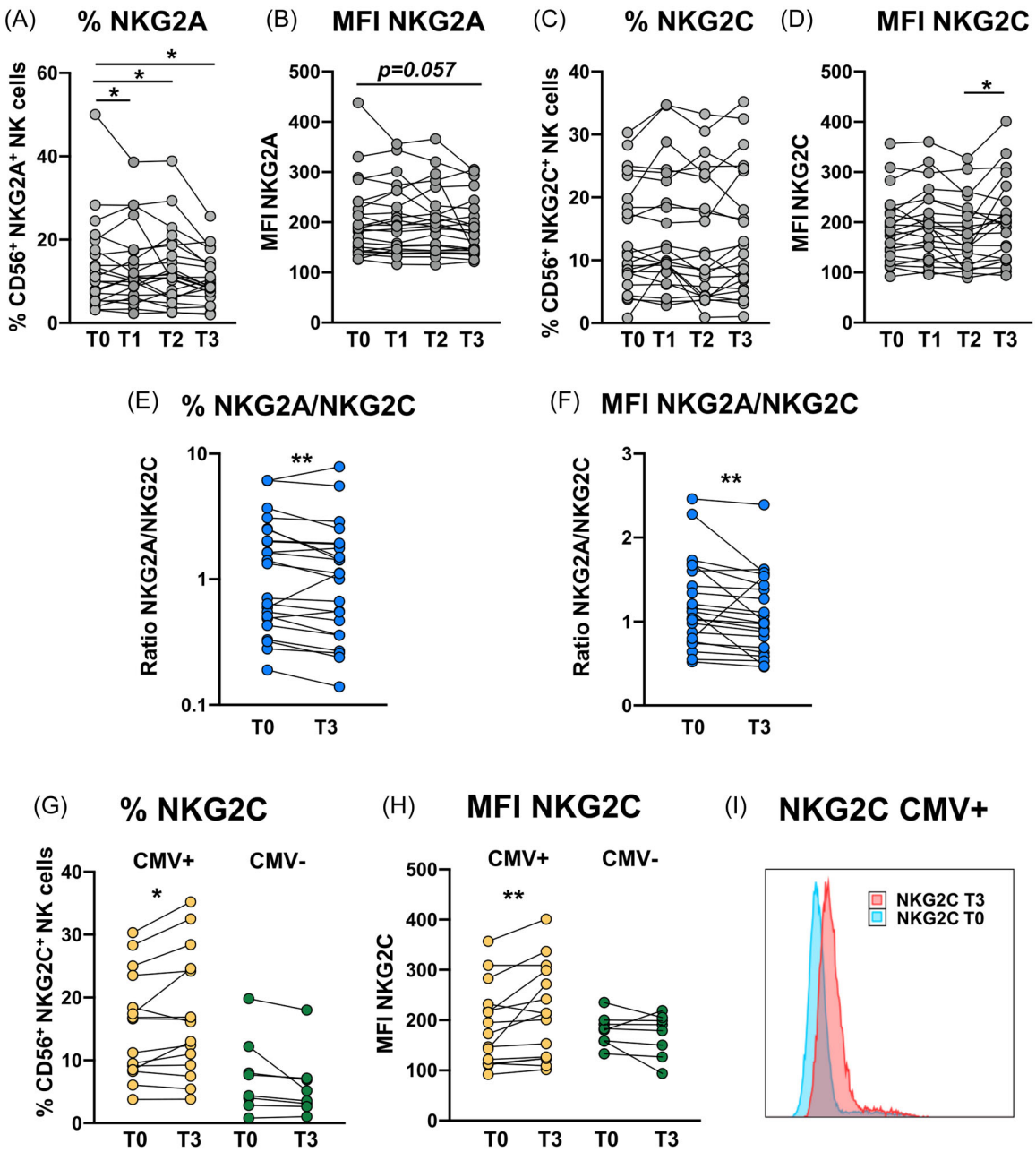

FIGURE 3 Reduced NKG2A/NKG2C ratio after BNT162b2 mRNA vaccination. Frequencies of (A) NKG2A + , (C) NKG2C+ and (E) NKG2A/NKG2C ratio and MFI of (B) NKG2A + , (D) NKG2C+ and (F) NKG2A/NKG2C ratio. (G) Frequencies and (H) MFI of NKG2C NK cells in CMV positive (yellow symbols) and negative (green symbols) individuals shown at T0 and T3. A representative overlay histogram of NKG2C at T0 and T3 is shown in I. The one-way Friedman with Dunn's multiple comparison test was used to compare data between the 4 timepoints. The non-parametric Wilcoxon signed-rank test was used to compare data between T0 and T3. *$p < 0.05$; ** $p < 0.01$.

4.4 | Late enhancement of antibody dependent cell-mediated cytotoxicity after booster

The enhanced ability of adaptive natural killer (NK) cells to perform antibody-dependent cellular cytotoxicity (ADCC) is well-documented.[9,32,33] Here we assessed ADCC in the presence of either SARS-CoV-2-infected or uninfected Vero E6 target cells from seven vaccinated individuals. To engage CD16 (FcγRIII) receptors on NK cells, we used autologous serum samples previously tested for the presence of SARS-CoV-2 S1/S2 Spike antibodies. Cell-surface expression of CD107a was used as a marker for NK cell degranulation, as a surrogate of ADCC. The data showed that CD107a degranulation was increased in the presence of serum containing spike-specific antibodies at T2 and T3 (Figure 7A). Comparison between timepoints showed increased degranulation at T3 compared with T0 in the presence of serum (Figure 7A). No differences between timepoints were observed in the presence of uninfected target cells. Interestingly, there was a decrease in the ability of NK cells to exert ADCC in the

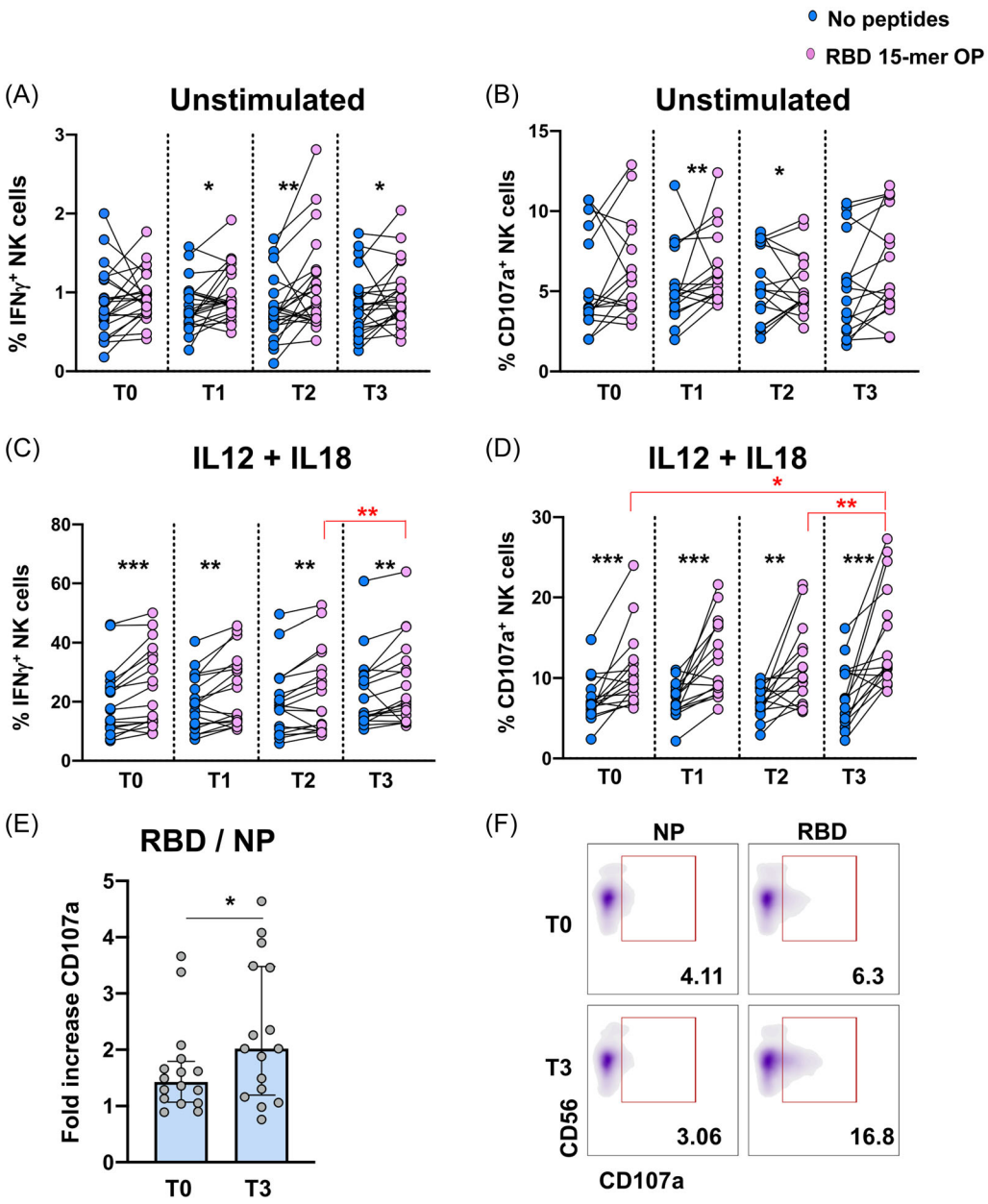

FIGURE 4 Changes in NK cell function induced by RBD overlapping peptides. (A) IFNγ production and (B) CD107a expression (degranulation) at T0, T1, T2 and T3 by unstimulated peripheral NK cells in the presence (pink symbols) or absence (blue symbols) of RBD peptides. (C) IFNγ and (D) CD107a expression in IL12 + IL18 stimulated NK cells. (E) Fold increase of the CD107a NK cell response for RBD stimulated compared with unstimulated (no peptides) cells at timepoints T0 and T3. (F) Representative dot plots of CD107a expression in IL12 + IL18 stimulated cells at T0 and T3. The one-way Friedman with Dunn's multiple comparison test was used to compare data between the 4 timepoints. The non-parametric Wilcoxon signed-rank test was used to compare data obtained in the presence or absence of RBD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

presence of SARS-CoV-2-infected cells (Figure 7A) compared with uninfected cells (Figure 7B). This finding confirms previously reported data which indicated that SARS-CoV-2 spike 1 protein reduced NK cell degranulation and induced NK cell exhaustion via HLA-E/NKG2A interaction.[34] Moreover, downregulation of ligands for the activating NKG2D is an escape mechanism from NK cell cytotoxicity in SARS-CoV-2 infected cells.[35]

ADCC experiments were also performed using pooled sera collected at timepoint T2 from five vaccinated subjects. Pooled sera were added to PBMC obtained at timepoints T0 and T3.

Cytolytic potential was equally induced at T0 and T3 timepoints, indicating that NK cells maintain the ability to kill infected cells in the presence of anti-Spike antibodies. However, CD107a expression was not significantly increased compared with T0. Interestingly, IFNγ

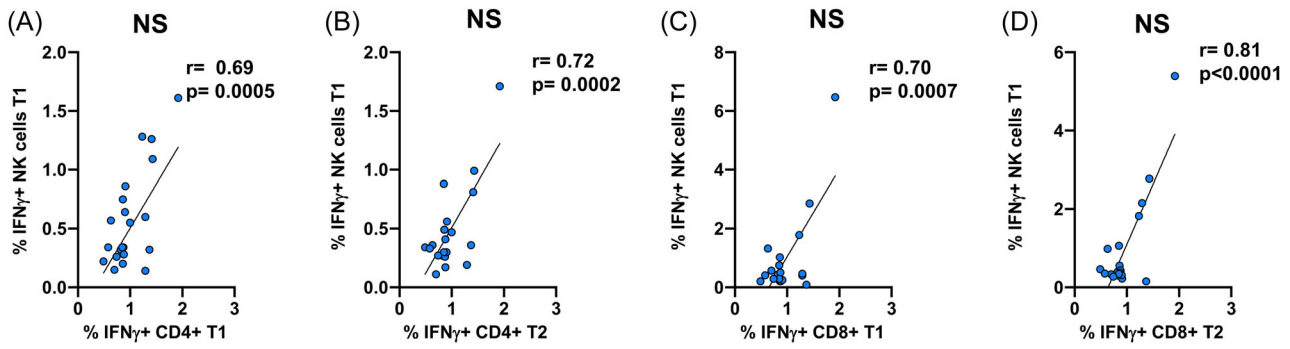

FIGURE 5 Positive correlations between innate and adaptive immune cells in vaccinees. Positive correlations between the frequencies of IFNγ + NK cells and IFNγ + CD4 + T cells at T1 (A) and T2 (B) with no cytokine stimuli. (C) Positive correlation between IFNγ + NK cells and IFNγ + CD8 + T cells at T1 (C) and T2 (D) without cytokine stimulation. The Pearson test was applied.

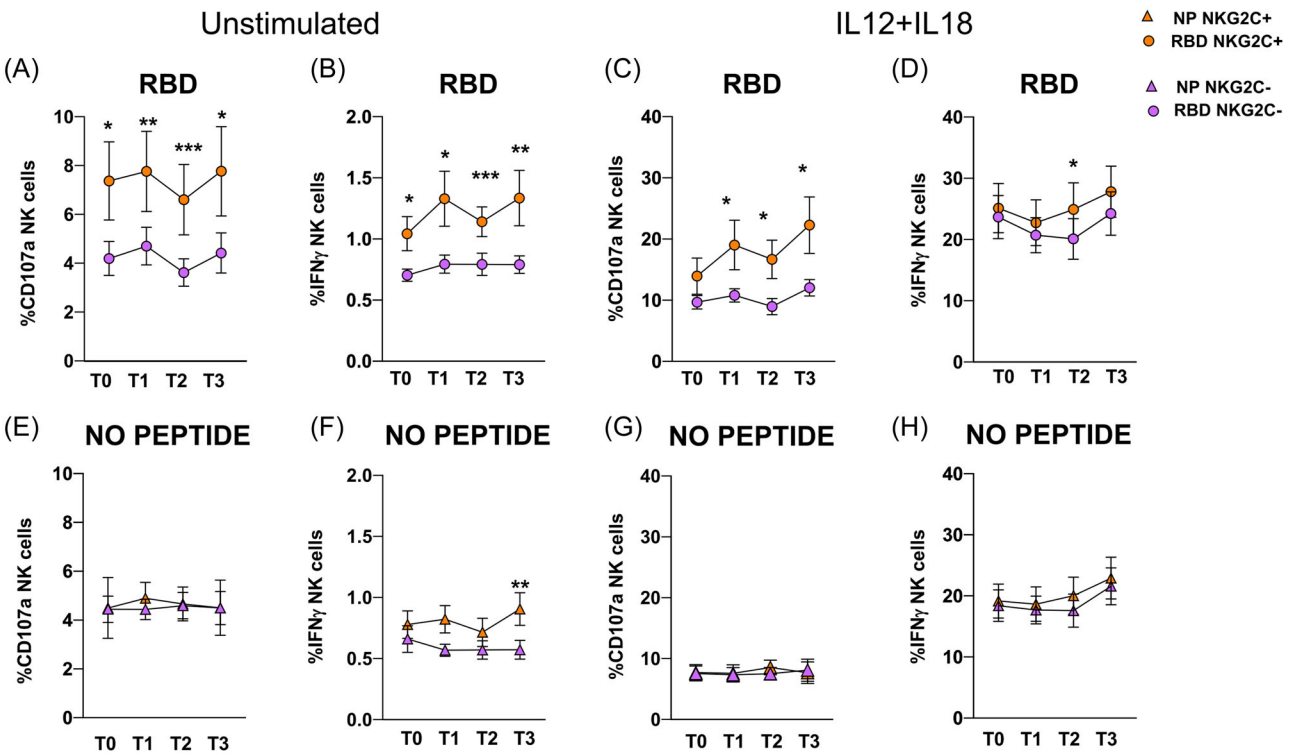

FIGURE 6 Increased IFNγ production and CD107a degranulation by NKG2C positive NK cells. CD107a degranulation and IFNγ production by NKG2C+ (orange dots) and NKG2C- (purple dots) NK cells following stimulation with RBD peptides, in the absence (A, B) or in the presence (C, D) of cytokine stimulation. CD107a degranulation and IFNγ secretion by NKG2C+ (orange dots) and NKG2C- (purple dots) NK cells without RBD peptides in the absence (E, F) or in the presence (G, H) of cytokine stimulation ($n = 15$). The non-parametric Wilcoxon signed-rank test was used to compare data $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

secretion was indeed significantly increased at T3 compared with T0 in the presence of pooled sera (Figure S9).

5 | DISCUSSION

Since the discovery of the SARS-CoV-2 vaccine, numerous studies have focused on humoral and adaptive immune responses. However, only a few have analyzed the innate immune cell compartment, particularly NK cells. In recent years, adaptive/memory NK cells have been identified, which may be involved in response to vaccines. Indeed, accumulating evidence suggests that NK cells play a role in both the induction and effector phases of vaccine-induced immunity against various viruses and other intracellular pathogens.[2,17–23]

However, only a few studies have focused on NK cells following SARS-CoV-2 vaccination. Here we showed that NK cells from individuals vaccinated with BNT162b2 vaccine enjoy higher effector function upon restimulation with SARS-CoV-2 RBD. Notably, NK cell

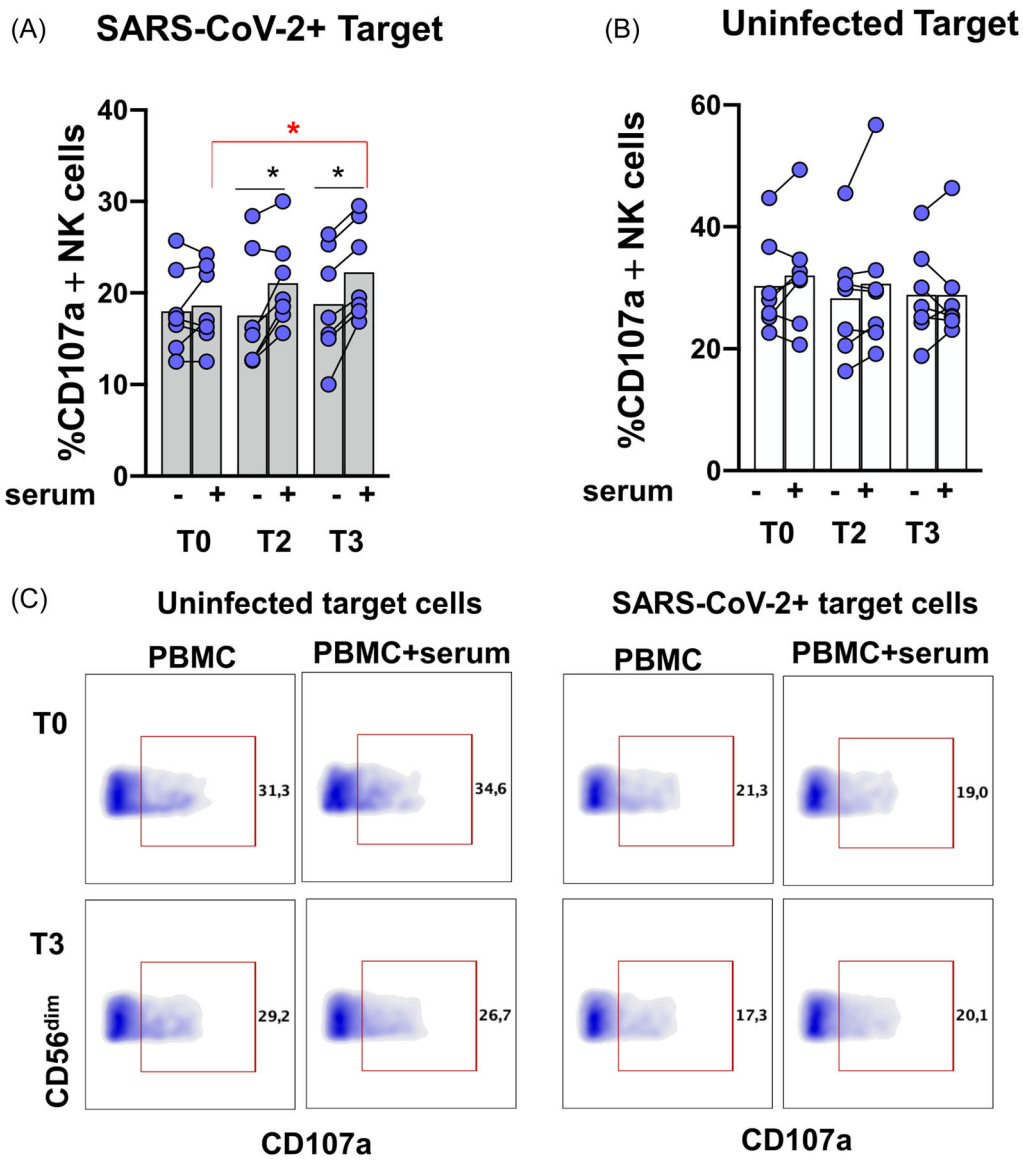

FIGURE 7 Antibody-dependent NK cell-mediated cytotoxicity. Frequency of CD107a+ NK cells cocultured with SARS-CoV-2 (A) infected or (B) uninfected Vero E6 cells in the presence or absence of autologous sera at T0, T2 and T3. (C) Representative dot plots of CD107a+ NK^{dim} cells after coculture with SARS-CoV-2-infected and uninfected Vero E6 target cells in presence or absence of autologous sera. Paired data between the same timepoint were analyzed by paired t test. Repeated Measure ANOVA followed by Dunnet's multiple comparison test were used to compare data among different timepoints. $^{*}p < 0.05$.

cytolytic potential was significantly increased several months after vaccination in response to RBD stimulation in the presence of cytokines, suggesting that a durable NK cell response may be induced upon subsequent antigenic and cytokine exposure. In line with this, ADCC activity in the presence of autologous sera was increased by RBD stimulation at T3 compared with baseline.

In agreement with our findings, Saresella et al.[36] found augmented IFNγ and CD107a expression by NK cells upon stimulation of PBMC with Spike protein during the follow-up period in BNT162b2 vaccinated individuals. Along the same lines, Cuapio et al. evaluated NK cell phenotype and functional capacity during the early phase (up to 35 days) after vaccination.[37] While they did not use SARS-CoV-2 antigens to stimulate cells, they reported preserved NK phenotype and function. In agreement with those findings, we observed a stable NK cell functional ability without stimulation with SARS-CoV-2 RBD peptides. However, in our phenotypic evaluation, we noted a reduction in the frequency of NK cells expressing NKG2A, a negative regulator of NK cell activity, 8 months after booster. In keeping with this, a reduction in the ILT2 inhibitory receptor and an increase in the KIR2DS2 activating receptor has been clearly shown in NK cells from vaccinated subjects over time.[36] Interestingly, NKG2A/NKG2C ratio was significantly reduced at the last timepoint, indicating a change in the balance between these HLA-E-interacting receptors, with a switch toward activation as a

function of time. Late NK cell activation after vaccination is also supported by the reduced TIM-3 expression observed at T3. TIM-3 is constitutively expressed on NK cells in their native state [38] and has been linked to mature or exhausted NK cell phenotypes.[39] Thus, collectively, these findings provide further evidence in support of vaccine-induced immunophenotypic changes in NK cells towards a more activated state.

It may be argued that the decision not to perform experiments with purified NK cells may not dismiss a possible role of adaptive immunity in NK cell activation rather than a direct stimulation of NK recall responses by RBD peptides. Indeed, our data using purified NK cells seem to indicate a supportive role of adaptive immunity in this context. If that is the case, it would be difficult to reconcile such a late NK cell response, that is, 9 months from baseline, much later than T and B cell responses, with a role of adaptive immunity in this setting. Alternatively, accessory cells, such as monocytes, may present SARS-CoV-2 peptides in the context of HLA-I eliciting NK recall responses. Interestingly, NK cell activity may be modulated by interaction of HLA-I loaded with peptides via their receptors. For instance, CMV peptides loaded on HLA-E can modulate activation of NK cells through binding with NKG2C,[11,12] while SARS-CoV-2 Nsp-13 peptide abrogate NKG2A-mediated inhibition, unleashing NK cell activity.[16] An experimental model in which antigen-loaded monocyte-derived dendritic cells (moDCs) were cocultured with purified NK cells has been used in other viral infections. Thus, Wijaya et al.[2] showed increased NK cytotoxic and proliferative responses using autologous hepatitis B surface antigen (HBsAg)-pulsed moDCs in HBV vaccinated individuals compared with unvaccinated subjects. Another study used NK cells isolated from livers of humanized mice previously vaccinated with HIV-encoded envelope protein, to show that HIV-Env–primed hepatic NK cells killed HIV-Env–loaded syngeneic target cells (DC), showing that NK cells displayed vaccination-dependent, antigen-specific recall responses in vitro.[40] While we acknowledge that it was not possible to explore the mechanistic details of peptide-induced NK cell activation in this context, our study clearly showed an increase in NK cell responses months postvaccination under physiological conditions.

NKG2C positive NK cells produced higher levels of IFNγ and expressed more CD107a compared with NKG2C negative NK cells after RBD stimulation, suggesting a possible specific role of memory NK cells in recognizing SARS-CoV-2 peptides. Interestingly, an association has been shown between NKG2C frequencies at baseline and antibody responses to BNT162b2 vaccine at day 35,[37] in agreement with data showing increased frequencies of NKG2C + NK cells in responders to influenza vaccination.[41] ADCC activity was slightly, though significantly, increased at the timepoints evaluated in RBD-stimulated NK cells. Notably, it has been shown that antibody-dependent NK cell function was strongly activated through non-spike antibodies in patients with SARS-CoV-2 infection, and this response was sustained for at least 6 months in most patients.[42]

In our study, a positive correlation between RBD-induced IFNγ in NK and CD4 + T cells was also observed, in support of a cooperation between innate and adaptive immunity against SARS-CoV-2. This is in line with a recent study [43] showing that the increased frequency of activated, CD69 + NK cells in SARS-CoV-2 vaccinated individuals after stimulation with spike protein, was CD4 + T cell dependent. Interestingly, a study in oncohematological patients showed that low serological responses to BNT162b2 vaccine was associated with reduced NK cell levels, indicating that NK cells play an active role in immune responses to vaccines.[44] Our study focused on BNT162b2 vaccine, as our subjects were enrolled at the very beginning of the vaccination campaign, when only Pfizer's Comirnaty vaccine was available. However, other studies have explored NK cell responses to various types of vaccines, revealing modulation in both phenotype and function. For instance, a study comparing the effects of heterologous (ChAdOx1-S/BNT162b2) and homologous (ChAdOx1-S/ChAdOx1-S) vaccination schedules found a significant down-regulation of CD16 exclusively in the group receiving the heterologous vaccine (ChAdOx1-S/BNT162b2).[45] Another study showed that NK cells exhibited potent ADCC function mediated by S2 antibodies after mRNA 1273 (Moderna) vaccination.[46] Other studies focused on patients with chronic virus infections. For instance, patients with chronic HBV infection showed a reduced frequency of NKG2A + NK cells and a concomitant decrease in HBsAg titers 30 days after BNT162b2 or ChAdOx1-S vaccination.[47] Another study analyzed the impact of Coronavac vaccine, based on inactivated virus, on NK cells in an HIV+ population and showed enhances vaccine-induced NK cell activation and degranulation in both healthy controls and HIV+ subjects.[48]

This study has some limitations. Firstly, the sample size is relatively small. However, it was necessary to carefully select healthcare workers who had not previously been exposed to SARS-CoV-2 to specifically investigate the vaccine's impact on NK cells. Secondly, it is important to emphasize that ADCC assays were conducted using autologous sera containing variable concentration of anti-Spike IgG. As a result, the quantitative aspects of ADCC may have been affected by differences in anti- Spike antibody titers across individuals. Moreover, we used Vero E6 cells as target cells in ADCC experiments because these cells are susceptible to SARS-CoV-2 infection. However, they are not an ideal model due to their origin from an African Green Monkey. In an effort to find a more suitable model, we tested the human epithelial cell line A549 expressing ACE2 (A549-ACE2 cells) as target cells. While these cells are also susceptible to SARS-CoV-2 infection, they do not express the Spike protein on their membrane 24, 48, and 72 h after virus inoculation, being useless for ADCC experiments. Figure S10 displays the expression of the Spike protein in both A549-ACE2 and Vero E6 cells, along with a representative ADCC experiment conducted using PBMC from individuals with a prior history of COVID-19 infection.

Despite these limitations, the study provides valuable insights into the vaccine's effects in this group of SARS-CoV-2 unexposed individuals, and the findings contribute to our understanding of NK cell responses to BNT162b2 vaccine.

In conclusion, our comprehensive analysis provides valuable insights into cellular immune responses induced by the BNT162b2 vaccine with implications for future vaccine development and

immunotherapeutic strategies. Understanding the specific mechanisms by which NK cells contribute to vaccine-induced immunity against SARS-CoV-2 can aid to design more effective and targeted approaches to combat viral infections.

AUTHOR CONTRIBUTIONS

Stefania Varchetta and Mario U Mondelli, conceptualized the study, wrote the article, supervised the team; Dalila Mele and Sabrina Ottolini provided substantial contribution to the conception and design of the study, acquisition, analysis and interpretation of data; Daniela Conteianni, Barbara Oliviero and Stefania Mantovani contributed to acquisition, analysis and validation of data; Andrea Lombardi, Alessandra Bandera, and Andrea Gori critically revised the manuscript and provided important intellectual input, Irene Cassaniti and Fausto Baldanti performed virological analyses and edited the manuscript. All authors critically read, edited, and approved the final version of the manuscript.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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