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CD4 and IL-2 mediated NK cell responses after COVID-19 infection and mRNA vaccination in adults

Amir M. Alhajjat * , Catherine R. Redden, Morgan Langereis, Steven T. Papastefan, Joy A.S. Ito, Katherine C. Ott, Lucas E. Turner, HeeKap K. Kang, Aimen F. Shaaban

Stanley Manne Children’s Research Institute, Department of Surgery, Ann & Robert Lurie Children’s Hospital of Chicago, Northwestern University, Chicago, IL, USA

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ABSTRACT

A detailed understanding of protective immunity against SARS-CoV-2 is incredibly important in fighting the pandemic. Central to protective immunity is the ability of the immune system to recall previous exposures. Although antibody and T cell immunity have gained considerable attention, the contribution of the NK cell compartment to immune recall and protection from SARS-CoV-2 has not been explored. In this study, we investigate the NK cell responses to stimulation with SARS-CoV-2 in previously exposed and non-exposed individuals. We show that NK cells demonstrate an enhanced CD4+ T cell dependent response when re-exposed to SARS-CoV-2 antigen. The enhanced response is dependent on T cells and correlates with the number of SARS-CoV-2 specific CD4 T cells. We find that IL-2 is a critical mediator of NK cell function. These findings suggest that NK cells contribute to the protective responses against SARS-CoV-2 through a cooperation with antigen-specific CD4 T cells and have significant implications on our understanding of protective immunity in SARS-CoV-2.

1. Introduction

Since January 2020, SARS-CoV-2 has spread throughout the world’s population causing widespread morbidity and mortality (WHO Coronavirus Disease Covid-19 Dashboard, 2021). Although social distancing, mask use and medical therapies have lessened the impact of the pandemic, control of the pandemic is ultimately achieved by protective immunity. Despite vaccines considerably decreasing the impact of SARS-CoV-2 in the world, our understanding of the protective immunological responses generated by vaccines remains in evolution. Protective immunity results from the cooperative interaction between multiple components of the immune system to effectively protect against infection (Hoebel et al., 2004). After vaccination, protective immunity is frequently gauged by humoral responses (Plotkin, 2010). Neutralizing antibodies provide some protection against secondary SARS-CoV-2 infection (Zost et al., 2020; Rogers et al., 2020; Khoury et al., 2021). However, neutralizing antibodies have not generally correlated with decreased disease severity. As such, transfer of antibodies after the infection has commenced provides limited benefit (Weinreich et al., 2021). In addition, SARS-CoV-2 antibody titers decay over time (Seow et al., 2020; Jo et al., 2021; Campo et al., 2021).

Nonetheless, cellular responses are capable of controlling viral infections even in the absence of antibodies. After hepatitis B infection and hepatitis B vaccination, immune protection persists despite antibody decline (Van Damme and Van Herck, 2007). Similarly, even in the absence of a humoral response, cellular responses are capable of controlling SARS-CoV-2 infection (Bange et al., 2021; Soresina et al., 2020). Fortunately, convalescent COVID-19 patients and BNT162b2 mRNA vaccine recipients develop robust T cell immunity lasting months (Dan et al., 2021; Zuo et al., 2021; Painter et al., 2021; Castellino et al., 2009). These memory T cells have an instrumental role in immune protection from COVID-19 in addition to control, reduction of severity and resolution of COVID-19 infection (Rydzynski Moderbacher et al., 2020).

Although memory has been generally considered the domain of T cells and B cells, NK cells have also been shown to contribute to memory responses both directly and indirectly. In the context of viral infections, NK cells can be activated by cytokines from antigen-specific CD4 T cells (Horowitz et al., 2010; Vargas-Inchaustegui et al., 2012; He et al., 2004), or display distinct antigen-specific memory features akin to T cells (Vivier et al., 2011; Marcus and Raulet, 2013). In either scenario, NK cells are capable of an enhanced recall response and serve protective roles against viral disease. The NK cell recall response to Influenza A

* Corresponding author.
E-mail address: aalhajjat@luriechildrens.org (A.M. Alhajjat).

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vaccination and the NK cell role in control of Simian Immunodeficiency Virus (SIV) are some examples (Vargas-Inchaustegui et al., 2012; Dou et al., 2015). Similar protective responses against SARS-CoV-2 would be valuable in COVID-19 immunity. However, despite numerous reports elucidating an important role for NK cells in both recovery and immunopathology of COVID-19, their role in protective immunity remains unknown.

In this study, we evaluate the NK cell responses to SARS-CoV-2 exposure in naïve and experienced settings. We examine their relationship with T cells and explore the role of IL-2 in the cooperative relationship between SARS-CoV-2 specific CD4 T cells and NK cells. We find that NK cells demonstrate a response to subsequent antigenic exposure that is dependent on CD3 T cells and proportionate to the Ag-specific CD4 T cell response and IL-2 production. We conclude that NK cells are effective mediators of the SARS-CoV-2 memory response elicited through a cooperation with antigen specific CD4+ T cells.

2. Materials and methods

2.1. Study individuals

Institutional review board approval was obtained from Ann & Robert Lurie Children’s hospital and Northwestern University IRB. Seventy-nine subjects were enrolled into the study with a total of 88 blood samples. All subjects gave written informed consent before being enrolled in the study. Participation was voluntary and compensated. Convalescent subjects (n = 18) were those who had recovered from confirmed SARS-CoV-2 infection documented by clinical RT-PCR results in addition to clinical symptoms consistent with COVID-19. None of the subjects had active disease at time of collection and were symptom free for at least 3 weeks. Thirty-one control subjects have never been diagnosed with COVID-19 or vaccinated. Individuals who have had any suspicious symptoms at any time after January 2020 were asked for documentation of a negative clinical COVID-19 test. Those not able to provide a negative clinical RT-PCR were excluded. Furthermore, presumed control subjects whom were found to have anti-SARS-CoV-2 specific antibodies were excluded due to the possibility of having an asymptomatic infection. Control individuals who were subsequently voluntarily vaccinated were brought back for interval evaluation. Vaccinated individuals were those who had recovered from confirmed SARS-CoV-2 infection documented by clinical RT-PCR results in addition to clinical symptoms consistent with COVID-19, their role in protective immunity remains unknown.

2.2. Blood sampling, preparation of PBMCs, cell culture and stimulation with recombinant protein

Blood was collected into EDTA coated vacutainers (BD). Samples were centrifuged at 1500g for 10 min to separate plasma from the cells. Plasma was stored at −80 °C for serological assessment as outlined below. PBMCs were then isolated using Ficoll gradient separation (Sigma-Aldrich). After washing with PBS, PBMCs were counted with a Countess automated cell counter (Thermo Fisher). In a 96 well plate (Thermo Fisher), 10^5 PBMCs were cultured in RPMI with 10 % fetal bovine serum, 1 % penicillin/streptomycin at 37 °C with 5 % CO2. Stimulation was achieved with addition of recombinant spike protein (1μg/well) (Sino Biological), or recombinant nucleocapsid protein at 1μg/well (Sino biological). Where indicated, blockade of IL-2 was done through addition of 10μg/ml of anti-human IL-2 neutralizing antibody (BD). After 22 h, culture plates were centrifuged, and the resultant supernatant was stored at −80 °C for cytokine analysis as detailed below. The cells were then washed twice and stained for flow cytometry. Data was acquired on a BD LSR II flow cytometer. Flow cytometry data was acquired on BD FACSDiva and analyzed using FlowJo v10.0. Staining was with the following antibodies: CD3 Pe-Cy5 (FN50), CD56 BV510 (NCAM), CD4 FITC (RPA-T4), CD8a Pe-Cy7 (RPA-T8), CD134 BV421 (Ber-Act35), CD69 PerCPCy5.5 (FN50) (Biolegend). NK cell activation was measured as percent NK cells (CD3- CD56^dim) expressing CD69+ above background levels measured in paired unstimulated control samples. CD56^bright NK cells in culture were prohibitively low in the culture and thus not analyzed. CD4 T cell activation was measured by measuring the percentage of CD4 T cells that are double positive for CD69 and OX40 above background levels measured in paired unstimulated control samples. All cultures were done in duplicate.

2.3. Analysis of anti-SARS-CoV-2 antibodies

ELISA for analysis of SARS-CoV-2 antibodies was performed as previously described (Amanat et al., 2020). 96 well plates (Nunc) were coated with 2 μg/ml of either S1 or Nucleocapsid (Sino) overnight at 4 °C. Plates were blocked with 3 % milk in PBS at room temperature for two hours and then washed with PBS with 0.1 % tween 20 (PBST). Plasma samples were prediluted diluted 1:5 with PBS in a separate plate then transferred into the test plate (1:100). Samples were then diluted further (1:300, 1:900) and incubated at room temperature for 2 h. The plate was then washed 3 × with PBST. 50 μl of anti-human IgG FAB HRP (Sigma) was added to each well and the plate was incubated for 1 h. Plates were washed 3 × with PBST. ELISA Substrate (Thermo Fisher) was added, and plates were developed for 10 min. The reaction was stopped using H2SO4 (Thermo Fisher). The plate was read at 450 nm absorbance using a BioTek Epoch 2 microplate reader (Biotek Instruments).

2.4. T cell depletion

Depletion of CD3 T cells was done using magnetic bead separation using manufacturer’s instructions. Briefly, PBMCs were washed and resuspended with Magnetic depletion buffer (Miltenyi Biotech). CD3 microbeads (Miltenyi Biotech) were then added for 15 min at 4 °C. The cells were then washed and resuspended in the buffer. Separation was then achieved by an Auto-MACS (Miltenyi Biotech) using depletes program. Samples were regarded as successfully depleted if the CD3 fraction was <.5 % of total lymphocytes.

2.5. Cytokine measurement with flow cytometry-based bead assay

Culture supernatant from the stimulation cultures were stored in 96 well plates at −80 °C. When ready for analysis, the supernatant was thawed and cytokines in the culture supernatant were measured using a flow cytometry bead-based assay (Human CD8/NK Kit, Biolegend) according to the manufacturer’s instructions. Briefly, the samples and mixed beads were incubated together in a 96 well filter plate (Biolegend) on a plate shaker for 2 h at 500 RPM. After two washes, biotin-labeled detection antibodies were added to each well and incubated on the plate shaker for 1 h at 500 RPM. Afterwards, Streptavidin-PE was added to each well for final incubation on the shaker for 30 mins at 500 rpm. Data was acquired on a Cytok Aurora (Cytex Biosciences). Analysis was then performed using the manufacturer’s LEGENDplex software (Biolegend).

2.6. Statistical analysis

Statistical analysis was done in GraphPad Prism (Version 9.1) and Microsoft Excel (Version 16). Statistical details are provided in the respective figure legend. Groups were compared using Mann-Whitney u-test. Correlation analysis was done using Pearson’s coefficient. NK cell and CD4 stimulation were calculated as background subtracted data which was derived by subtracting the stimulation in the no stimulation wells from the stimulation in the corresponding recombinant protein well. Experiments were always done in duplicate. Cytokine data was also measured as background subtracted data which was derived by subtracting the cytokine concentration in the no stimulation wells from the stimulation in the corresponding recombinant protein well.
3. Results

3.1. Clinical characteristics and SARS-COV-2 antibody response of study cohort

Seventy-nine individuals participated in the study. One peripheral blood sample was obtained from most although (9/81) were sampled more than once for longitudinal assessment as outlined in each experiment. Clinical characteristics of study participants can be found in Fig. 1, Table 1 and supplemental Tables I-III. Thirty-one individuals without a known previous history of COVID-19 or vaccination were included. These were considered control subjects. Nine of the control individuals also voluntarily received the BNT162b2 vaccine and were evaluated again after receiving the vaccine. Eighteen individuals were drawn after recovery from COVID-19 and were symptom free for at least 3 weeks. Median time between positive PCR and blood draw for convalescent individuals was 65 days (range 32–210 days). None of the convalescent donors experienced severe disease and none were hospitalized. One patient was asymptomatic, 8/18 were mild infections while 9/18 had moderate severity as defined by NIH COVID criteria (Spectrum, 2021). Eighteen individuals were drawn after receiving the BNT162b2 mRNA vaccine. Nine blood draws were done 5–14 days (Median: 12 days) after the first dose of the vaccine (Time point 1 (T1)). Sixteen blood draws were 5–22 days (Median: 15.5 days) after receiving the second dose (Time point 2 (T2)). Four blood draws were done 62–82 days (Median 63 days) after the second dose of the vaccine (Time point 3 (T3)) and ten blood draws 106–205 days (Median: 188 days) after the second dose of the vaccine (Time point 4 (T4)). anti-SARS-CoV-2 (Receptor Binding Domain (RBD) specific IgG, Spike (SP) specific IgG and Nucleocapsid (NC) specific IgG) antibodies were measured at every blood sampling and demonstrated consistent with current literature. SP and RBD-specific IgG were significantly higher in convalescent and vaccinated individuals when compared to controls. Vaccinated individuals demonstrated levels of NC-specific IgG similar to controls whereas convalescent individuals had higher levels of NC-specific IgG. A slight decay in RBD and Spike specific IgG was seen in T4 individuals highlighting the reduction in humoral immunity months after vaccination consistent with current literature (Seow et al., 2020; Jo et al., 2021; Campo et al., 2021). Seroconversion was seen in all control participants who were additionally tested after vaccination (supplemental Fig. 1).

3.2. NK cells from convalescent and vaccinated individuals demonstrate antigen-specific recall-type responses

The NK cell response to secondary antigen exposure in convalescent and vaccinated individuals was compared to controls using an in vitro antigen stimulation assay to stimulate peripheral blood mononuclear cells (PBMCs) with SARS-CoV-2 recombinant SP. After overnight stimulation, the frequency of activated NK cells (CD3 CD56dimCD69+) was measured using multi-parameter flow cytometry as illustrated in Fig. 2. CD69 expression has been shown to correlate with cytotoxic potential of NK cells (Donskoi et al., 2011; Borrego et al., 1999; Clausen et al., 2003). As shown in Fig. 3, convalescent (13.59 % ± 3.73) and vaccinated (T1: 13.38 % ± 2.61, T2: 13.90 % ± 3.55, T3: 18.45 % ± 7.56, T4: 22.75 % ± 5.78) individuals demonstrated a significant increase in the frequency of activated NK cells after SP exposure when compared to control individuals (0.90 % ± 0.18). There were no significant differences in the frequency of activated NK cells between convalescent and vaccinated individuals. When measured prior to and after vaccination, all vaccinated individuals exhibited a significantly higher NK cell response to SP after vaccination (1.39 % ± 0.44 pre-vaccine versus 20.37 % ± 5.83 post-vaccine, p < 0.0001) (Fig. 3b). Additionally, the durability of the recall response in NK cells from convalescent and vaccinated individuals was surprisingly evident >6 months after primary antigen exposure even after antibody levels had started to fall (Fig. 3a and supplemental Fig. 2).

Unlike convalescent individuals, vaccinated individuals have not been exposed to SARS-CoV-2 NC as the BNT162b2 mRNA vaccine only encodes the SP (Walsh et al., 2020). Therefore, if secondary NK cell responses are linked to previous exposure to SARS-CoV-2 antigens, vaccinated individuals would not be expected to demonstrate secondary responses to NC. To determine if the observed recall responses were antigen-specific, the NK cell responses to NC was compared between the groups. As demonstrated in Fig. 3c, convalescent individuals (21.03 % ± 4.52) demonstrated a significant increase in activated NK cells following NC stimulation when compared to vaccinated (T1: 1.00 % ± 0.59, T2: 0.52 % ± 0.18, T3: 5.57 % ± 1.44, T4: 4.16 % ± 1.21) or control individuals (4.69 % ± 1.28). As expected, the frequency of activated NK cells responding to NC stimulation was unchanged after vaccination (6.29 % ± 2.64 pre-vaccine versus 5.26 % ± 1.84 post-vaccine, p > 0.99) (Fig. 3d). Notably, some control and vaccinated individuals demonstrated a slight increase in the frequency of activated NK cells in response to NC likely due to cross-reactivity from prior infection with other coronaviruses (Weiskopf et al., 2020; Ogbe et al., 2021). Collectively, these findings indicate that NK cells previously exposed to the SARS-CoV-2 virus or mRNA vaccine demonstrate durable antigen-
specific recall-type responses to subsequent SARS-CoV-2 antigen exposure.

### 3.3. SARS-CoV-2 NK cell recall response is T cell-dependent

NK cell memory has been shown to occur via direct NK cell receptor binding to cognate antigens and through indirect activation following antigen-specific humoral or T cell responses (Horowitz et al., 2010; Vargas-Inchaustegui et al., 2012; He et al., 2004; Vivier et al., 2011; Marcus and Raulet, 2013). To better understand the mechanisms regulating NK cell recall responses to SARS-CoV-2 antigens, CD4 T cell responses to secondary viral antigen exposure were measured. Following

| Characteristics of study individuals. | Control | Convalescent | Vaccinated |
|--------------------------------------|---------|--------------|------------|
| **Age Median (Range)**               | 37 (23–67 years) | 34.5 (23–62 years) | 43 (26–56 years) | 39.5 (23–58 years) | 34 (26–43 years) | 37 (23–67 years) |
| **Sex**                              | 30 (96.77 %) | 15 (83.33 %) | 4 (44.44 %) | 9 (56.25 %) | 4 (100 %) | 8 (80.00 %) |
| Female                               | N/A     | N/A          | N/A        | N/A        | N/A        | N/A        |
| Male                                 | 1 (3.23 %) | 3 (16.66 %) | 5 (55.55 %) | 7 (43.75 %) | 0 (0 %) | 2 (20.00 %) |
| **Median days to Blood Draw (Range) from PCR or vaccination (as applicable)** | 65 (32–210) days | 12 (5–14) days | 15.5 (5–22) days | 63 (62–82) days | 188 (106–205) days |
| **Number of Participants**           | 31      | 18           | 9          | 16         | 4          | 10         |
| **COVID-19 symptom Severity**        | N/A     | 1 (5.55 %)   | N/A        | N/A        | N/A        | N/A        |
| Asymptomatic                         | N/A     | 8 (44.44 %)  | N/A        | N/A        | N/A        | N/A        |
| Mild                                 | N/A     | 9 (50.00 %)  | N/A        | N/A        | N/A        | N/A        |
| Moderate                              | N/A     | 9 (50.00 %)  | N/A        | N/A        | N/A        | N/A        |
| **Race**                             | 26 (83.80 %) | 16 (88.88 %) | 5 (55.55 %) | 14 (87.50 %) | 4 (100.00 %) | 10 (100.00 %) |
| Caucasian                            | 26 (83.80 %) | 16 (88.88 %) | 5 (55.55 %) | 14 (87.50 %) | 4 (100.00 %) | 10 (100.00 %) |
| Non-Caucasian                        | 5 (16.13 %) | 2 (11.11 %) | 3 (33.33 %) | 2 (12.50 %) | 0 (0 %) | 0 (0 %) |
| Other                                | 0 (0 %) | 0 (0 %) | 1 (11.11 %) | 0 (0 %) | 0 (0 %) | 0 (0 %) |
| **Report having long-term health condition(s)** | Yes | 11 (35.48 %) | 11 (61.11 %) | 2 (22.22 %) | 3 (18.75 %) | 1 (25.00 %) | 3 (30.00 %) |
| No                                   | 20 (64.52 %) | 7 (38.89 %) | 7 (77.77 %) | 13 (81.25 %) | 3 (75.00 %) | 7 (70.00 %) |

Fig. 2. Flow cytometric analysis. Gating strategy and representative histograms and dot plots used for analysis after in vitro stimulation of PBMCs with SARS-CoV-2 antigens Spike (SP) and Nucleocapsid (NC) in control, convalescent and vaccinated individual.
Fig. 3. Experienced NK cells demonstrate recall to stimulation with SARS-CoV-2 antigen. PBMCs from controls, convalescent and vaccinated participants were left unstimulated (media alone) or stimulated with recombinant Spike protein (SP). After overnight culture, cells were stained for surface markers and analyzed using flow cytometry. Expression of CD69 on CD56<sup>dim</sup> CD3<sup>-</sup> NK cells was measured. (A) NK cell response after stimulation with the SP in controls, convalescent and vaccinated individuals was measured as frequency of CD69 positive cells among CD56<sup>dim</sup> CD3<sup>-</sup> NK cells (Control, n = 31; Convalescent, n = 18; T1, n = 9; T2, n = 16; T3, n = 4; T4, n = 10). (B) Frequency of CD69 positive cells among CD56<sup>dim</sup> CD3<sup>-</sup> NK cells measured after SP stimulation in individuals prior to and after receiving the vaccine. Post vaccine timepoints are combined (n = 9). (C) NK cell response after stimulation with the NC in controls, convalescent and vaccinated individuals was measured as frequency of CD69 positive cells among CD56<sup>dim</sup> CD3<sup>-</sup> NK cells (Control, n = 31; Convalescent, n = 18; T1, n = 9; T2, n = 16; T3, n = 4; T4, n = 10). (D) Frequency of CD69 positive cells among CD56<sup>dim</sup> CD3<sup>-</sup> NK cells measured after NC stimulation in individuals prior to and after receiving the vaccine. Post vaccine timepoints are combined (n = 9). T1: 5–14 (median:12) days after first dose of BNT162b2 vaccine; T2: 5–22 (median:15.5) days after completion of two doses of the vaccine; T3: 62–82 (median:63) days after completion of two doses of the vaccine; T4: 106–205 (median:188) days after completion of the two doses of the vaccine. Stimulation was calculated as background subtracted data which was derived by subtracting the stimulation in the no stimulation wells from the stimulation in the corresponding recombinant protein well. Cultures were always done in duplicate. Significance was determined by two-tailed Mann-Whitney U-test. Error bars represent SEM.

Fig. 4. Frequency of SARS-CoV-2 antigen specific CD4 T cells in control, convalescent and vaccinated individuals. Expression of CD69 and OX40 on CD4 T cells was measured after culture with SARS-CoV-2 SP or NC antigen. Antigen-specific CD4 T cells were defined as those positive for both CD69 and OX40. (A) Antigen specific CD4 T cells after stimulation with SP in controls, convalescent, and vaccinated individuals. (B) Antigen specific CD4 T cells after stimulation with the NC in controls, convalescent individuals measured as CD69 and OX40 positive CD4 T cells. (Control, n = 31; Convalescent, n = 18; T1, n = 9; T2, n = 16; T3, n = 4; T4, n = 10) T1: 5–14 (median:12) days after first dose of BNT162b2 vaccine; T2: 5–22 (median:15.5) days after completion of two doses of the vaccine; T3: 62–82 (median:63) days after completion of two doses of the vaccine; T4: 106–205 (median:188) days after completion of the two doses of the vaccine. Stimulation was calculated as background subtracted data which was derived by subtracting the stimulation in the no stimulation wells from the stimulation in the corresponding recombinant protein well. Cultures were always done in duplicates. Significance was determined by two-tailed Mann-Whitney U-test. Error bars represent SEM.
in vitro stimulation with recombinant SP, cytotoxic CD4 T cells were identified by co-expression of both CD69 and OX40 (Porichis et al., 2014; Reiss et al., 2017). The gating scheme and representative samples of the expression are demonstrated in Fig. 2. As demonstrated in Fig. 4a, significantly more SP-reactive cytotoxic CD4 T cells were identified in convalescent (0.636 ± 0.05) and vaccinated (T1: 1.173 ± 0.308, T2: 0.707 ± 0.181, T3: 0.929 ± 0.282, T4: 1.400 ± 0.336) individuals when compared to controls (0.146 ± 0.054). There was also no significant difference in the frequency of cytotoxic CD4 T cells between convalescent and vaccinated individuals. As expected, NC-reactive CD4 T cells were only detected in convalescent (0.448 ± 0.137) individuals but not controls (0.114 ± 0.037) or vaccinated (T1: 0.126 ± 0.046, T2: 0.062 ± 0.048, T3: 0.128 ± 0.103, T4: 0.173 ± 0.067) individuals (Fig. 4b). These findings are consistent with the current understanding of the CD4 T cell responses to SARS-CoV-2 antigens in convalescent and vaccinated individuals (Dan et al., 2021; Zuo et al., 2021; Painter et al., 2021).

The relationship between the frequency of SP-reactive cytotoxic CD4 T cells and NK cell recall responses to SARS-CoV-2 antigens in convalescent and vaccinated individuals was examined. As shown in Fig. 5a, the NK cell recall response correlated with the CD4 T cell response to SP in both convalescent and vaccinated groups when stimulated with SP for both groups (r = 0.52, p < 0.0001, n = 61). To uncouple NK cell responses from T cell responses, CD3+ T cells were depleted from the cytotoxic assays prior to stimulation with SP and compared to non-depleted cytotoxic assays for the convalescent, vaccinated and control groups (Supplemental Fig. 3). As shown in Fig. 5b, the NK cell recall response was strikingly diminished by the loss of T cells in the convalescent (pre-depletion 9.45% ± 2.85 versus post-depletion 0.56% ± 0.34, p = 0.029) and vaccinated groups (pre-depletion 30.59% ± 9.53 versus post-depletion 0.58% ± 0.21, p = 0.029). Conversely, T cell depletion did not alter the naïve NK cell response seen in control individuals (pre-depletion 0.93% ± 0.36 versus post-depletion 0.89% ± 0.84, p = ns) (Fig. 5b). Taken together, these findings suggest that NK cell recall responses to SARS-CoV-2 antigens are dependent on T cell activation.

3.4. SARS-CoV-2 NK cell recall response is mediated by IL-2

Rapid secretion of IL-2 by activated antigen-specific CD4 T cells is a known pathway for indirect activation of NK cells (Horowitz et al., 2010; He et al., 2004). We thus assessed the importance of IL-2 in the NK cell response to SARS-CoV-2. First, the levels of IL-2 were measured in culture supernatants from vaccinated individuals when stimulated with SP for both groups (T1: 157.9±68.2 pg/ml, T2: 87.92±25.19 pg/ml, T3: 56.56±31.46 pg/ml, T4: 256.9±128.0 pg/ml) individuals when compared to control individuals (1.21±1.9 pg/ml). Furthermore, the NK cell response in both convalescent and vaccinated individuals correlated directly with the level of secreted IL-2 in the culture supernatants (Fig. 6b). IL-2 secretion was nearly undetectable in culture supernatants from controls and vaccinated participants when stimulated with NC (Supplemental Fig. 4). An analysis of multiple proinflammatory cytokines revealed a broad elevation in the secreted levels in convalescent (with SP and NC stimulation) and vaccinated individuals (only with SP stimulation) when compared to controls (Supplemental Fig. 5).

To further examine the role of IL-2 in the NK cell response, we cultured fresh PBMCs from vaccinated individuals with SP in presence of IL-2 neutralizing antibody and measured the resultant NK cell activation. Neutralization of IL-2 was confirmed by flow cytometric bead assay. As shown in Fig. 6c and d, there was a significant reduction in the NK cell response to SP stimulation, although this was not statistically significant. This data suggests a significant role for IL-2 as a critical mediator for the NK cell response to SARS-CoV-2. Alternatively, a neutralization of IL-2 may have inhibited CD4 T cell responses leading to further decrease in NK cell responses.

4. Discussion

In this study, we demonstrate an enhanced NK cell response following secondary exposure to the SARS-CoV-2 virus that is dependent on antigen-specific T-cells. Secreted levels of IL-2 appeared to be an important mediator in the NK cell response. These findings reveal a role for NK cells in immunologic memory against SARS-CoV-2 and offer a new method to track sophisticated mechanisms of cellular immunity after vaccination.

NK cells as effectors of innate immunity play a fundamental role in clearance of viral infections (Jost and Altfeld, 2013). An increased susceptibility to viral infections is evident in conditions of diminished NK cell frequency or function (Jawahar et al., 1996). Similarly, normal NK cell function is an important component of the primary response to SARS-CoV-2 whereas defects in NK cell immunity contribute to the disease spectrum (Maucourant et al., 2020; Kramer et al., 2021; Littera et al., 2021; Witkowski et al., 2021). Early evidence for a critical role for NK cells stems from findings in which diminishing numbers of NK cells correlated with increasing severity of SARS-CoV-2 disease (Masselli et al., 2020; Vabret et al., 2020; Ni et al., 2021), a phenomenon also reported in respiratory disease caused by closely related coronaviruses (He et al., 2005; National Research Project for SARS, Beijing Group,

![Fig. 5](image-url) NK cell recall response is T cell dependent. (A) Correlation between frequency of CD69+OX40+CD4 T cells and CD69+ NK cells when stimulated with Spike (SP) in convalescent and vaccinated individuals (r = 0.52; p < 0.0001; n = 61). (B) NK cell response measured as frequency of CD69 positive cells among CD56dim CD3- NK cells with and without depletion of CD3 cells in controls (n = 4), convalescent (n = 4), and vaccinated subjects (n = 4). Non-parametric Spearman test was used for correlation analysis. For group comparison, significance was determined by two-tailed Mann-Whitney u-test.
More recent findings have demonstrated a correlation in severity of SARS-CoV-2 disease with the expression of particular subsets of NK cells and receptors (Zheng et al., 2020).

Given the importance of NK cells in acute SARS-CoV-2 infection, a protective NK cell role in SARS-CoV-2 immunity after infection or vaccination is possible and may be mediated by several mechanisms. Notably, NK cells have been shown to harbor explicit antigen-specific memory characteristics in contexts such as hapten-induced contact hypersensitivity (O’Leary et al., 2006), murine cytomegalovirus (Sun et al., 2011), and cytokine induced memory NK cells (Cooper et al., 2009). In humans, NK cell memory has been recognized in human CMV, herpes simplex virus, influenza A and hantavirus although the exact mechanisms remain elusive (Abdul-Careem et al., 2012; Lopez-Vergès et al., 2011; Bjorkstrom et al., 2011; Wijaya et al., 2021; Nikzad et al., 2019; Stary et al., 2020). The results of the current study reveal enhanced NK cell responses in subjects previously exposed to SARS-CoV-2 through either illness or vaccination. The finding that NK cells from vaccinated individuals responded to SP, but not NC, supports that the cooperative CD4 T cell antigen exposure to antigen. This was corroborated by the interval assessment of CD4 NK cell response is specific and requires prior CD4 T cell antigen presentation (O’Leary et al., 2006), murine cytomegalovirus (Sun et al., 2011), and cytokine induced memory NK cells (Cooper et al., 2009). In humans, NK cell memory has been recognized in human CMV, herpes simplex virus, influenza A and hantavirus although the exact mechanisms remain elusive (Abdul-Careem et al., 2012; Lopez-Vergès et al., 2011; Bjorkstrom et al., 2011; Wijaya et al., 2021; Nikzad et al., 2019; Stary et al., 2020). The results of the current study reveal enhanced NK cell responses in subjects previously exposed to SARS-CoV-2 through either illness or vaccination. The finding that NK cells from vaccinated individuals responded to SP, but not NC, supports that the cooperative CD4 T cell antigen exposure to antigen. This was corroborated by the interval assessment of CD4 NK cell response is specific and requires prior CD4 T cell antigen exposure to antigen. This was corroborated by the interval assessment of CD4 NK cell responses in vaccinated individuals (T1: n = 4, T2: n = 16, T3: n = 4, T4: n = 7). IL-2 production was significantly increase in convalescent and vaccinated individuals when compared to controls. (B) Correlation of IL-2 and frequency of CD69+ among CD56dimCD3- NK cell when stimulated with SP. (C) Frequency of CD69+ among CD56dimCD3- NK cell when PBMCs are stimulated with SP with and without neutralizing IL-2 antibodies. (D) Percent reduction in and frequency of CD69+ among CD56dimCD3- NK cell with and without neutralizing IL-2 antibodies and stimulation with SP (n = 10). Post vaccine timepoints are combined (n = 11). T1: 5–14 (median: 12) days after first dose of BNT162b2 vaccine; T2: 5–22 (median: 15.5) days after completion of two doses of the vaccine; T3: 62–82 (median: 63) days after completion of two doses of the vaccine; T4: 106–205 (median: 188) days after completion of the two doses of the vaccine. IL-2 concentration was calculated as background subtracted data which was derived by subtracting the stimulation in the no stimulation wells from the stimulation in the corresponding recombinant protein well. Experiments were always done in duplicates. Significance was determined by two-tailed Mann-Whitney u-test. Error bars represent SEM. Non-parametric Spearman test was used for correlation analysis. LOD: Limit of detection. ADCC in SARS-CoV-2 immunity.

NK cells may also respond indirectly through NK cell cooperation with antigen specific CD4 T cells in control of viral infections. Such CD4 T cell-mediated NK cell functions have been exhibited in the context of hepatitis B virus, influenza A vaccination, rabies and simian immunodeficiency virus (Horowitz et al., 2010; Vargas-Inchaustegui et al., 2012; He et al., 2004). Following exposure to these antigens through infection or vaccination, antigen-specific IL-2 expression by memory CD4 T cells enhances NK cell activation and facilitates clearance of the virus. In rabies vaccinated individuals and SIV, NK cell memory functions have been found to completely depend on T cell-derived IL-2 whereas NK cell memory is elicited in HBV vaccinated individuals despite absence of T cell derived IL-2 (Vargas-Inchaustegui et al., 2012; Wijaya et al., 2021). The findings of the current study revealed that enhanced NK cell responses in SARS-CoV-2 are also mediated by IL-2 expression by memory CD4 T cells. Neutralizing IL-2 antibody only reduced the response indicating a partial but significant role for IL-2 among SARS-CoV-2 vaccinated individuals. This interplay enhances the effectiveness of the antigen-specific adaptive immune response in protection from SARS-CoV-2 infection. Indeed, links between the adaptive and innate immune responses are vital in host defense and allow for amalgamation of the specificity of the adaptive immune response with the rapidity of the innate immune response.

Another potential advantage of memory NK cell responses in viral disease is the broadened response to SARS-CoV-2 variants.
studies in subjects that received seasonal influenza vaccine demonstrate an increased and sustained NK cell recall response to multiple influenza virus subtypes despite antibody-mediated responses that were not as broad (Dou et al., 2015). Interestingly, these memory NK cells demonstrated a dynamic increase in intracellular Nkp46 which correlated with enhanced IFN-γ response (Dou et al., 2015). Although none of the vaccinated volunteers in the current study reported breakthrough infections prior to the emergence of the omicron variant, future studies are needed to examine NK cell responses to SARS-CoV-2 variants.

Alternatively, NK cells have also been found to have a suppressive effect on adaptive immune responses after vaccination. Mechanisms involved include NK cell clearance of infected and antigen bearing cells, perforin-dependent NK cell killing of activated CD4 and CD8 T cells and production of immunosuppressive cytokines (Rydzynski et al., 2015; Cox et al., 2021). These immunomodulatory effects of NK cells can be protective from immunopathology related to viral infections or detrimental to vaccination efforts.

Collectively, these findings reveal a critical role for NK cells in protective immunity against SARS-CoV-2. Weaknesses of the current study include that intracellular cytokines were not measured and the cytotoxic effect of NK cells was not directly measured. Rather we chose the activation status of NK cells demonstrated by the CD69 expression as a surrogate for enhanced activity. Therefore, an exact contribution of the activated NK cells to anti-SARS-CoV-2 immunity is not well defined. As such, further studies are needed to define whether these activated NK cells contribute to enhanced viral killing versus immunomodulation of the adaptive immune response. Additionally, further studies are also needed to characterize the mechanisms that guide NK cell responses in COVID-19. On a practical level, the NK cell response to SP antigen may provide a method for evaluation of vaccine efficiency to provide broad immunity against different viral subtypes and potentially guide booster intervals.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.imbio.2022.152204.

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