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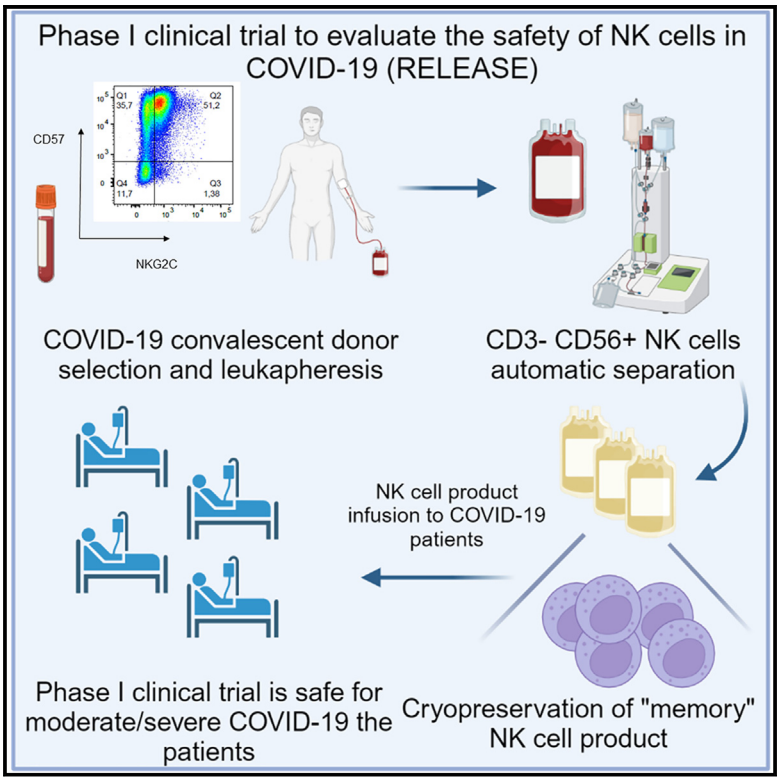
The phase I RELEASE clinical trial to evaluate the safety of NK cells in COVID-19

Graphical abstract



Highlights

- NK “memory” from convalescent donors as an adoptive therapy for COVID-19 patients (RELEASE)
- Clinical trial assessed NK cell infusion safety, immune recovery, and patient outcomes
- No serious adverse events reported
- Clinical trial found no dose-limiting toxicities after NK cell infusion

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In brief

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The phase I RELEASE clinical trial to evaluate the safety of NK cells in COVID-19

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SUMMARY

The severity of COVID 19 symptoms has a direct correlation with lymphopenia, affecting natural killer (NK) cells. SARS-CoV-2 specific "memory" NK cells obtained from convalescent donors can be used as cell immunotherapy. In 2022 a phase I, dose-escalation, single center clinical trial was conducted to evaluate the safety and feasibility of the infusion of $CD3^{-}/CD56^{+}$ NK cells against moderate/severe cases of COVID-19 (NCT04578210). Six participants with pneumonia and/or lymphopenia were infused. Four patients received a single-dose infusion of NK cells of 1×10^{6}/kg, and the following two patients a dose of 2×10^{6}/kg of NK cells. All participants' clinical status and inflammation markers were monitored. No serious adverse events were reported after infusion. Exploratory outcomes included the donor chimerism, NK-cell immunophenotype evolution, and immune lymphocyte reconstitution. This study provides preliminary evidence supporting the idea that treatment of COVID-19 patients with moderate/severe symptoms using NK from COVID-19 convalescent donors is feasible and safe.

INTRODUCTION

Since December 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread worldwide, causing a global pandemic. The virus causes coronavirus 2019 disease (COVID-19), which can manifest a variety of clinical symptoms, ranging from asymptomatic infection or mild influenza-like syndrome, to acute pneumonia, multi-organ failure and death.[1] This range of symptoms is largely due to risk factors, such as age or comorbidities, but it is also determined by

the heterogeneous response of each individual's immune system.[2]

Lymphopenia has a direct correlation with disease severity and is an independent marker of mortality.[3] Lymphocytes play a key role in maintaining immune homeostasis and inflammatory response[4] and can affect natural killer (NK) cells, which are essential for countering viral infections, including SARS-CoV-2.[5] Although immunological memory has been attributed to the adaptive immune system, a subpopulation of NK cells also has memory properties and a higher capacity to respond to certain stimuli.[6]

Current treatment of the infection is based on antiviral and steroidal anti-inflammatory drugs, anticoagulants, optimal oxygen therapy support, and in severe presentations, cytokine blockade. Although vaccine administration has drastically reduced severe and critical forms of the disease,[7] there are still patients who could benefit from alternative treatments, such as immunotherapy. We hypothesized that SARS-CoV-2 specific "memory" NK cells from convalescent donors could be used for treating these patients.

We previously studied the presence of a SARS-CoV-2 "memory" NK cell population in the blood of convalescent unvaccinated donors. We concluded that the presence of a $CD57^+$/$NKG2C^+$ double-positive population within the NK cells had a close correlation with the acquisition of memory properties.[8] These cells can be stored and are immediately available as an "off-the-shelf" COVID-19 convalescent donor-derived biobank.[9]

The RELEASE trial (NCT04578210) is a phase I/II clinical trial that uses adoptive cell therapy based on NK cells or memory T cells derived from convalescent donors who overcame COVID-19, with the goal of phase I to evaluate the safety, feasibility, and recommended phase II dose (RP2D) of a single infusion.[10]

Here, the outcomes of the memory NK arm of the RELEASE phase I clinical trial are reported. The results of the $CD45RA^-$ T cell arm of the study have been reported in previous manuscripts.[11,12]

RESULTS

Patient characteristics

Between January and July 2022, six participants were enrolled in the trial. The first four patients received a single-dose infusion of NK cells of 1×10^6/kg, and the following two patients a dose of 2×10^6/kg. Two patients were lost to follow-up after 2 weeks (#2 from cohort 1, and #5 from cohort 2). The trial was terminated without completing the expected recruitment because global COVID-19 vaccination resulted in a lower incidence of pneumonia. Given the difficulties in recruiting patients for cell therapy trials, we consider it important to report the outcomes of these 6 participants.

The included patients were five adult men and one adult woman, with a median age of 69 years (IQR [Interquartile Range] 51.25–75), with moderate SARS-CoV-2 pneumonia and/or lymphopenia. Their main comorbidities were hypertension (two patients) and dyslipidaemia (four patients). Furthermore, three patients were ex-smokers. One patient (#5) had severe heart disease (severe aortic stenosis and atrial fibrillation), severe heart failure, hypoxia, and lymphopenia but no pneumonia (Table 1). In addition, four patients had complete vaccination status (two or three vaccines), according to the immunization schedule of Spanish authorities at the time. Patient #1 had received one dose, and only patient #2 had not received a vaccine against SARS-CoV-2. Patient #6 was diagnosed with concomitant influenza A infection during hospitalization.

The median S/F ratio (SpO2/FiO2) at time of infusion was 261 (IQR 236.3–301.5), corresponding to a mild adult respiratory distress syndrome.[13] All six patients received the infusion of NK cells in the first 24 h of hospitalization. Following the trial design, four patients received a low dose (cohort 1) and two patients received an intermediate dose (cohort 2). The main clinical characteristics and outcomes, comorbidities, clinical status, and concomitant treatment for COVID-19 are shown in Table 1.

Outcomes

All patients underwent the treatment within the first 10 days of symptom onset (median 7.25, IQR 4.15–10). All six patients required oxygen supplementation. Two patients (#1 and #6) were admitted to the intermediate respiratory care unit (IRCU) for high-flow nasal cannula and non-invasive ventilatory support. None of the patients required invasive ventilatory support. The median hospital stay after the infusion was 6.5 days (IQR 2.75–12.75).

Five patients received corticoids (dexamethasone), at a median cumulative dose of 38 mg (IQR 20–149), and three patients were administered remdesivir. Higher doses of dexamethasone were administered to the two patients who were admitted to the IRCU. Heparin was administered to five patients, and the remaining patient continued outpatient therapy with apixaban. No biological therapy or experimental drugs for COVID-19 were administered.

The results of the clinical and analytical monitoring are shown in Table 2. The clinical status, according to the National Early Warning Score (NEWS), had a median score of 4 (IQR 4–4.25) at screening. The NEWS has been shown to be a robust predictive clinical scoring system to identify deterioration in infected patients outside the intensive care unit.[14]

We also monitored the patients' clinical index with the use of a 7-level ordinal scale.[15] Our cohort had a median score of 4 (IQR 3.75–4) at screening. Only patient #6 had an increase on this scale (on day 7) and a slower recovery. Patient #3 had a more rapid improvement by day 3. The other four patients had a gradual decrease after day 7, when reported (Table 2).

Respiratory status was monitored by recording oxygen saturation and flow, using the S/F ratio. Patients #1 and #6 had significant worsening by day 3, were admitted to the IRCU, and presented a slower recovery. Patients #3 and #5 registered an improvement by day 3. Patient #2 had a progressive recovery until day 7 (last data available), and patient #4 was required domiciliary oxygen at discharge.

Laboratory results were also monitored. At screening, three patients had severe lymphopenia, with a median lymphocyte count of 1080/mcL (IQR 645–1387.5). Patients #2, #3, and #4 had a rapid recovery by day 3, while patients #1, #5, and #6 had an initial worsening of the lymphocyte count. In patient #1,

Table 1. Baseline characteristics of participants in the arm 2 phase I RELEASE clinical trial

Natural killer cells infused/kg	1 × 10^6	1 × 10^6	1 × 10^6	1 × 10^6	2 × 10^6	2 × 10^6
Patient number	#1	#2	#3	#4	#5	#6
Gender	Male	Male	Male	Male	Male	Female
Weight (kg)	81	65	73	80	65	38
Age (years)	72	58	74	66	78	31
Comorbidities						
Arterial hypertension	Yes	No	No	No	Yes	No
Dyslipidaemia	Yes	No	Yes	Yes	Yes	No
Diabetes mellitus	No	No	No	No	No	No
Chronic heart disease	No	No	No	No	Yes	No
Chronic respiratory disease	No	No	No	No	No	Yes
Smoking	Ex-smoker	Never	Never	Ex-smoker	Ex-smoker	Never
Others	Nephrolithiasis	–	Parkinson's, Prostate adenocarcinoma	Hypothyroidism	Aortic valve stenosis, atrial fibrillation	Mastocytosis, Rett's syndrome, influenza A coinfection
Vaccination status	Yes: Moderna (1)	No	Yes: Pfizer (3)	Yes: Astra-Zeneca (2) + Pfizer (1)	Yes: Pfizer (3)	Yes: Janssen (1) + Pfizer (1)
Treatment						
Dexamethasone (mg)	Yes (194)	Yes (38)	Yes (12)	Yes (28)	No	Yes (104)
Remdesivir (mg)	No	Yes (600)	No	Yes (600)	No	Yes (600)
Heparin	Yes	Yes	Yes	Yes	No[a]	Yes
S/F at infusion	333	253	239	228	269	291
NEWS score at infusion	4	4	4	4	5	4
7-point score at infusion	4	4	3	4	4	4
Time (days) from disease onset to hospital admission	10	8	5	10	2	7
Time (days) at intermediate respiratory care unit	9	0	0	0	0	4
Time (days) of hospitalization after infusion	14	5	3	8	2	11
Time (days) to SARS-CoV-2 negative PCR after infusion	6	Unknown	Unknown	Unknown	Unknown	1
Outcome	Pulmonary embolism	Recovery	Recovery	Pulmonary Fibrosis	Recovery	Recovery
Alive 12 months	Yes	Yes	Yes	Yes	Yes	Yes
Long COVID symptoms	No	No	No	Yes	No	No

[a]Oral anticoagulation with apixaban.

the lymphopenia persisted in all the available records (until day 14).

We observed a general decrease in proinflammatory parameters. The median C-reactive protein level at screening was 65.9 mg/dL (IQR 19.15–104.37). In line with their clinical and respiratory status, patients #1 and #6 had temporary worsening, followed by a subsequent progressive decrease. Similarly, there was a general reduction in all the inflammatory parameters (Table 2).

Records of the time needed for a negative SARS-CoV-2 result by PCR were available for two patients. Patient #1 had a negative result on day 6, and patient #6 had a negative result on day 1 post-infusion. Data on the remaining patients were not available.

Safety

The primary outcome was to determine the dose-limiting toxicity (DLT) to define the RP2D for a phase II trial. There were no immediate reaction complications in any of the infusions. AEs were defined as potential risks associated with NK cells infusion, which may include infusion reactions, side effects related to frozen products, graft-versus-host disease and unpredictable complications, as described in the study protocol.[10] They were monitored for the first month (when available) by clinical and analytical assessment.

No AEs were reported, including transfusion-related acute lung injury, cytokine release syndrome, graft-versus-host disease, fever, or worsening of respiratory failure, and there was

Table 2. Clinical and analytic outcome

Natural killer cells infused/kg	1×10^6	1×10^6	1×10^6	1×10^6	2×10^6	2×10^6
Patient number	#1	#2	#3	#4	#5	#6
Clinical outcome						
S/F						
Pre	333	253	239	228	269	291
Day 3	164	341	496	291	390	189
Day 7	326	337	–	319	–	289
Day 14	360	–	496	337	–	431
Day 21	481	–	–	337	–	–
Day 28	481	–	–	337	–	–
NEWS score						
Pre	4	4	4	4	5	4
Day 3	2	4	0	6	1	2
Day 7	2	3	–	2	–	3
Day 14	1	–	0	1	–	–
Day 21	0	0	–	1	–	–
Day 28	0	0	–	1	–	–
7-point score						
Pre	4	4	3	4	4	4
Day 3	4	4	1	4	3	4
Day 7	3	2	–	3	–	5
Day 14	1	–	1	1	–	3
Day 21	1	1	–	1	–	–
Day 28	1	1	–	1	–	–
Laboratory findings						
Lymphocytes × 10^9/L						
Pre	630	1300	650	1290	870	1650
Day 3	650	2240	1800	–	730	640
Day 7	930	4100	–	5070	–	1770
Day 14	900	–	1420	3180	–	440
Day 21	–	2850	–	–	–	–
Day 28	–	3040	–	2460	–	–
C-reactive Protein mg/L (NR 0–5)						
Pre	106.7	90.9	103.6	21.1	13.3	40.9
Day 3	124	21.1	68.6		28.8	43.3
Day 7	38.6	9	–	0.8	–	11.7
Day 14	6.8	–	1.6	22	–	5.3
Day 21	–	2.7	–	26.1	–	–
Day 28	–	2.1	–	15	–	–
Fibrinogen, mg/dL (NR 150–450)						
Pre	523	824	535	592	572	561
Day 3	613	580	470	673	–	419
Day 7	408	556	–	348	–	–
Day 14	275	–	277	471	–	375
Ferritin, ng/mL (NR 10–291)						
Pre	4001	750	188	–	–	–
Day 3	3970	555	183	278	142	29
Day 7	3294	1014	–	289	–	–
Day 14	2587	–	173	–	–	–
D-dimer, ng/mL (NR < 500)						
Pre	2760	550	340	420	–	–
Day 3	1160	300	280	410	28,8	560
Day 7	1260	570	–	380	–	–
Day 14	720	–	480	320	–	–
LDH, U/L (NR 100–190)						
Pre	424	417	257	273	–	249
Day 3	421	297	133	218	290	344
Day 7	288	305	–	311	–	–
Day 14	264	–	–	–	–	242
ALT (U/L) (NR < 35)						
Pre	64	36	15	26	–	52
Day 3	42	37	9	21	32	31
Day 7	48	275	–	34	–	19
Day 14	38	–	26	45	–	46
AST (U/L) (NR < 40)						
Pre	63	53	29	30	–	38
Day 3	63	31	22	17	27	24
Day 7	34	145	–	29	–	17
Day 14	26	–	20	34	–	34

Abbreviations: NEWS, National Early Warning Score; LDH, lactate dehydrogenase; ALT, alanine transaminase; AST, aspartate aminotransferase.

no significant alteration in liver enzymes (Table 2). DLT was not identified with doses up to 1×10^6/kg after one month. However, patient #1 was diagnosed 2 months later with pulmonary embolism, and patient #4 was discovered underlying pulmonary fibrosis, over unknown previous emphysema, requiring oxygen supplementation after hospital discharge, although we consider these events related with COVID-19 known complications.

Chimerism, immune reconstitution, and NK cell immunophenotype evolution

Within the exploratory outcomes, immune reconstitution and chimerism were evaluated (Tables 3 and S1). Chimerism is defined as the percentage of donor NK cells that are detected in the patient after infusion. It allows us to know that cells infused are alive and viable, and how long they last in the patient's body. Overall, there was an increase in the number of lymphocytes, from 0.69×10^9/L (IQR 0.35–0.99) at recruitment to 1.82×10^9/L (IQR 1.17–2.29) at the best recovery point after infusion. Of the six patients infused with NK cells, #1 and #4 presented the best recovery point one-week post-infusion, #2 at three weeks post-infusion, and #3 at two weeks post-infusion. Post-infusion data from patients #5 and #6 were not documented (Figure 1).

The best recovery scenario for patients #1, #2, #3, and #4 resulted in recovery in the $CD3^+$, $CD4^+$, and $CD8^+$ cells. Regarding

Table 3. Best recovery after infusion

Natural killer cells infused/Kg	1×10^6	1×10^6	1×10^6	1×10^6	2×10^6	2×10^6
Patient number	#1	#2	#3	#4	#5	#6
Pre-infusion						
T ($CD3^+$) cells $\times10^9$/L	0.338	0.942	0.363	0.722	0.658	1.173
$CD4^+$ cells $\times10^9$/L	0.251	0.535	0.077	0.514	0.472	0.59
$CD8^+$ cells $\times10^9$/L	0.072	0.367	0.281	0.171	0.112	0.503
B cells $\times10^9$/L	0.067	0.355	0.04	0.24	0.039	0.398
NK cells $\times10^9$/L	0.22	0.001	0.236	0.327	0.164	0.077
NKT (%)	0.79	0.88	31.81	4.1	1.32	5.98
Best recovery after infusion						
T ($CD3^+$) cells $\times10^9$/L	1.448*	2.204**	1.082***	2.33*	ND	ND
$CD4^+$ cells $\times10^9$/L	1	1.406	0.483	1.825	ND	ND
$CD8^+$ cells $\times10^9$/L	0.39	0.717	0.581	0.41	ND	ND
B cells $\times10^9$/L	0.373	0.235	0.037	1.455	ND	ND
NK cells $\times10^9$/L	0.574	0.406	0.251	0.62	ND	ND
NKT (%)	15.66	8.1	24.79	2.28	ND	ND

n/d no data; * day 7; ** day 21; *** day 14.
Immune recovery of $CD3^+$, $CD4^+$, $CD8^+$, $CD56^+$, $CD19^+$ cells, and NKT subpopulation after infusion of NK cells

B cells, these were diminished in patient #2 and remained at the same level in patient #3. NK T cell percentage decreased in patient #3 (Table 3). NK-cell numbers showed recovery in patients #1, #2, and #4. Table 3 shows the percentage of various T cell subpopulations and B-cell memory cells, which were generally maintained in patient #1, with a slight increase in the $CD8^+$ $CD45RA^-$ population and a decrease in the B-cell memory population. In patient #2, the $CD45RA^+$ population drastically decreased after infusion, whereas the $CD45RA^-$ population increased. The B cell memory population was also highly reduced after the infusion.

Donor micro chimerism was followed for four weeks after infusion of NK cells. As shown in Table 4, micro chimerism was detected in patients #1, #2, and #3 in the first week after infusion. Patient #4's micro chimerism was not detectable until two weeks after infusion. All the patients shared at least one allele with the donor, and in all cases, a killer immunoglobulin-like receptor (KIR) mismatch between donor and patient was found (Table S2).

The NK-cell immunophenotype was studied on days 0, 3, 7, 14, and/or 28 after infusion (Figure S1). Patients #1, #2, and #4 were studied at all time points. There was no consistent pattern in the expression of the analyzed markers among the patients.

Tim-3 receptor behavior differed drastically among the patients. Its presence was undetectable in patient #1, strongly present in patient #2, and oscillated in patient #4. Moreover, NK cytotoxic receptors such as NKp30 and NKp46 were more present in patient #1 than in patients #2 and #4. NKG2A inhibitory marker was poorly expressed among all patients at all-time points. Lastly, there appeared to be a strong correlation between perforin granulocytes and EOMES (eomesodermin) transcription factor expression in all the patients.

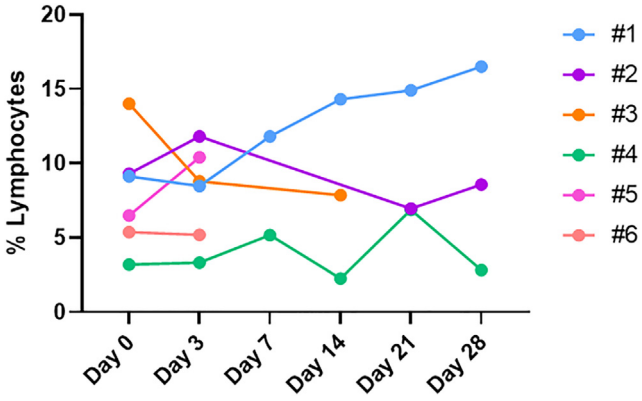

Figure 1. Lymphocyte recovery

Cytokine profile after Natural killer cell infusion

There was a decreasing tendency of IL-22, IL-2, IL-6, and IL-10 levels (Figure S2). IL-13 was below the threshold level and was not detected. The main limitation of the study was the absence of all the "paired time point" measurements. Only in 2 patients were we able to obtain serum for all the time points.

Natural killer cell function and phenotype in presence of dexamethasone

After performing a two-way ANOVA, we concluded that there were no significant differences in the interferon (IFN)-γ production due to dexamethasone at 10^{-7} M doses, although there was a tendency toward progressively lower production of IFN-γ at higher concentrations and over time (72 h) (Figure S3A).

A comparison analysis was also performed between the total number of NK cells and dexamethasone. There were no differences in the number of cells with clinically employed doses of dexamethasone (10^{-7} M) at 24 h, although there was a significant reduction of cells at 72 h ($p = 0.01$) (data not shown).

Finally, checking the activation phenotype, no differences were observed for human leukocyte antigen class II (HLA-DR) or CD69 receptors due to dexamethasone. In the case of the CD25 receptor, there were differences between 24 h and 72 h for dexamethasone concentrations of 10^{-7} M and 10^{-5} M ($p = 0.04$ and $p = 0.018$, respectively) (Figure S3B). Regarding the total number of NK cells, again there were no differences due to dexamethasone; however, the number of NK cells were significantly reduced after 72 h (*$p = 0.01$; **$p = 0.001$; ***$p < 0.0001$) (Figure S3C).

DISCUSSION

Safety

This study showed the outcomes of six hospitalized patients with COVID-19 pneumonia and/or lymphopenia requiring oxygen supplementation after the administration of a single dose "off the shelf" NK cell infusion from a convalescent COVID-19 donor. This study was a phase I clinical trial focused on dose and schedule determination and patient safety, with a conventional 3 + 3 design. The product development process is feasible, cost-effective, and easy to develop in centers with experience in hematopoietic stem cell transplantation.[8]

Table 4. Donor chimerism after infusion of NK cells from the convalescent donor

Natural killer cells infused/kg	1×10^6	1×10^6	1×10^6	1×10^6	2×10^6	2×10^6
Patient number	#1	#2	#3	#4	#5	#6
Donor chimerism (%)						
Day 0–7	0.8	0.02	0.5	0	ND	ND
Day 7–14	<0.01	–	<0.01	0	ND	ND
Day 14–21	<0.01	<0.01	–	0.02	ND	ND
Day 21–28	<0.01	<0.01	–	0.02	ND	ND

The indicated chimerism values were obtained as the mean value of the analysis of 2 different INDEL systems. Pre-infusion patient samples were analyzed, and results were employed as negative background control. The data are shown by weeks after infusion.

Although the RELEASE phase I study[11] was designed to evaluate the safety of sequentially escalating doses of NK cells (cohort 1, 1×10^6 cells/kg; cohort 2, $1–5\times10^6$ cells/kg; cohort 3, 5×10^6 cells/kg to 1×10^7 cells/kg), given that our supply of NK cells was obtained from two donors, the doses of the original RELEASE trial were modified, with calculated progressive doses of 1×10^6 cells/kg (cohort 1), 2×10^6 cells/kg (cohort 2), and 3×10^6 cells/kg. Due to the decreasing incidence of the disease at the time of enrollment, recruitment was completed without reaching our goal (18 patients) because of the lack of eligible candidates, nor was a RP2D established. However, none of these COVID-19 patients developed any serious AEs after treatment. Similar results were observed in our previous experience with T lymphocyte infusion,[11,12] supporting the safety of cell infusion therapy. Adoptive NK-cell treatment displays no toxicity, and the cell product can be stored in a lymphocyte biobank to provide "off the shelf" access for subsequent viral pandemics[9].

This study emphasizes the difficulties in recruiting patients for cell therapy trials, although NK-cell infusion was found to be safe and feasible. However, no conclusions can be drawn regarding efficacy because of the small number of included patients and insufficient biological markers.

We observed none of the general AEs associated with cryopreserved cell products in DMSO, such as nausea, vomiting, and abdominal cramps. Numerous factors can contribute to adverse reactions caused by DMSO, such as age, weight, sex, specific diseases, the procedure, speed of injection, and time from thawing to injection.[16]

Clinical and analytical outcomes

Four patients had sustained recovery at day 28 and had at least one year of follow-up. Patient #1 had persistent dyspnoea, was diagnosed with pulmonary embolism shortly after discharge, and underwent anticoagulant therapy until October 2022. At the 1-year follow-up, he was fully recovered and showed no persistent symptoms, as did patients #2, #3, #5, and #6. Patient #4 had persistent dyspnoea, with the diagnosis of pulmonary fibrosis and emphysema during hospitalization, requiring periodic follow-up. These complications are well known in the clinical outcomes of SARS-CoV-2 pneumonia and do not appear to have any correlation with the cell infusion therapy.[17]

In comparison with the cohort of our phase I study with memory T cell infusions[11] performed in 2020, our cohort was slightly older (median of 69 vs. 59 years), had more comorbidities, and had a milder clinical presentation, probably due to their vaccination status. The S/F score at screening indicated a slightly poorer respiratory status (291 T cells vs. 261 NK cells) but with a more rapid recovery, once again because of their vaccination status. Proinflammatory parameters were also slightly lower than in the T cell cohort (median 65.9 vs. 76.95 mg/L).

The global median lymphocyte counts at recruitment increased from 0.69×10^9 to 1.82×10^9 cells/L at the best recovery time point. The degree of lymphopenia (global or by subpopulation) is related to disease severity and can even predict mortality, which is more marked between days 7 and 9 of illness and has proven to be independent of other risk factors.[18] In this cohort, patients #1 and #6, who had a decrease in their baseline lymphocyte count, had a poorer clinical progression; patient #3, who had severe initial lymphopenia, recovered clinically and analytically by day 3.

Exploratory outcomes

We evaluated the effect of dexamethasone treatment on NK-cell function and phenotype. Corticoids are well-known inhibitors of NK function.[19] In our evaluation, however, in the presence of SARS-CoV-2 peptides, the administration of dexamethasone at an equivalent dose of 6 mg does not affect IFN-γ production. Regarding phenotype, cell activation was not affected by dexamethasone either.

The percentage of NK cells available from a peripheral blood sample is approximately 3%–10%, with an ultimate efficiency of 50% of these cells. Compared with our previous investigation with CD45RA lymphocyte infusion in which we were capable of obtaining 20–40 aliquots,[20] the number of aliquots for NK infusion obtainable from one donor is much lower (1–3).

Donor-recipient selection was not performed prior to infusion, although it was subsequently studied (Table S2). Every patient missed at least one KIR ligand in relation to the donor, resulting in KIR ligand incompatibility or KIR mismatch.[21] It is known that this particular immune situation mediated by alloreactive NK cells confers a more positive outcome in neoplastic scenarios and hematopoietic transplantation[22] and could do so against viral infections.

Regarding immune reconstitution after NK-cell infusion in our cohort, lymphocyte counts increased at one, two, or three weeks after infusion, as observed in the memory T cell arm of the RELEASE clinical trial.[11] This outcome was similar to the ones achieved in other clinical trials in which NK cells were infused.[23] Donor micro-chimerism was found in three patients in the first week after infusion; and in one of the patients two weeks after infusion, which is poorer compared with T cell micro chimerism.[12] This event could occur due to the short life span of NK cells[24] and the deficient response to cryopreservation. To solve this last issue, we employed a cryopreservation medium that was previously tested for NK cells.[9] Nevertheless, there is still room for improvement in this matter.

Table 5. Clinical trials using natural killer cell therapy in COVID-19 and other viral infections

NCT number	Disease	Treatment	Country	Status
NCT04900454	COVID-19	Allogeneic NK cell therapy derived from $CD34^+$ hematopoietic stem cells	USA	Completed No publications available
NCT04280224	COVID-19	Conventional treatment plus twice weekly NK cells ($0.1–2\times10^7$ NK cells/kg)	China	Completed No publications available
NCT04365101	COVID-19	Human placental hematopoietic stem cell-derived natural killer cells (CYNK-001)	USA	Active, not recruiting https://doi.org/10.1158/1538-7445.AM2021-CT201
NCT04324996	COVID-19	NK cells, IL15-NK cells, NKG2D CAR-NK cells, ACE2 CAR-NK cells, NKG2D-ACE2 CAR-NK cells	China	Unknown No publications available
NCT04634370	COVID-19	Phase I clinical trial on NK cells for COVID-19	Brazil	Unknown No publications available
NCT04531319	COVID -19	Immune cell groups	Turkey	Completed No publications available
NCT05258643	COVID -19	The role of natural killer, complement and t-lymphocytes in covid-19 disease, a prospective monocentric study (TONIC)	Belgium	Recruiting No publications available
NCT04344548	COVID-19	Adoptive cell transfer for the immunotherapy of COVID-19	Colombia	Withdrawn No publications available
NCT04320303	CMV Viremia Transplantation Infection	Donor-derived expanded NK cells	China	Unknown. No publications available
NCT03899480	HIV Infections Immune Deficiency	Haploidentical natural killer cells and *N*-803	USA	Completed. No publications available
NCT02961712	HTLV-1-associated myelopathy	NK cells and amniotic epithelial cells	China	Unknown No publications available

We studied the immunophenotype of the patients' NK cells at various time points after transfusion. We observed no clear pattern in the increase, decrease, or maintenance of the expression of the various markers among patients. Due to the challenges of the pandemic, compiling the samples at all-time points from all patients was a difficult task; thus, we lack a complete scenario. NK "memory" cells, determined by the presence of NKG2C and CD57 markers,[8] increased from day 3 after transfusion. Given that there was not a strong microchimerism of administered NK cells, this increase could be the result of the patients' capacity to develop their own NK "memory" cells against SARS-CoV-2 infection.

NK cell therapy

Due to their specific features, NK cells are currently being studied in several experimental trials to boost the immune system against various diseases. Several approaches to NK-based therapies have gained interest, including immune stimulants, with promising clinical results in the treatment of malignancies.[25] NK cells also play a key role in fighting viral infections.[26] NK cell-based therapy has been explored in influenza A infection,[27] in controlling human adenovirus reactivation, and against human herpesvirus-6B encephalitis in allogeneic hematopoietic stem cell transplantation.[28]

Given that, under normal conditions, NK cells are expected to effectively destroy infected macrophages and other infected cells that are responsible for causing cytokine storms, their lower circulating counts and exhausted phenotype could contribute to a more severe presentation of COVID-19. Thus, NK-cell reconstitution or adoptive transfer therapy of properly functioning NK cells is an attractive therapeutic choice.[29] However, their potential application for treating viral infections has yet to be evaluated in large-scale clinical trials.

There are several clinical trials using NK cell-based products and NK-cell stimulants to fight COVID-19. Table 5 summarizes the current clinical trials that use NK cell therapy in various viral infections, including COVID-19, reflecting the growing interest in this therapeutic approach. There are very few currently available publications regarding these trials' results.

There is evidence that NK cells play a key role in the cellular immune response against SARS-CoV-23.[5] The infusion of *ex vivo*-expanded allogeneic NK cells has been reported to be safe and feasible.[30] To our knowledge, this is the first report of human passive adoptive cell therapy using NK memory cells from convalescent COVID-19 donors.

As in many COVID-19 clinical trials, due to the declining incidence of the infection in relation to vaccination campaigns, recruitment in this study was completed without reaching our goals, nor did we establish a phase II recommendable dose. Follow-up of the patients was incomplete mainly due to the epidemiological circumstances and the difficulty in attending visits after hospital discharge, assuming subsequent medical stability. Nonetheless, at the infusion dose of 1×10^6 cells/kg clinical and analytical data were collected, without reporting any AE. Therefore, although monitoring was suboptimal, we consider DLT was not identified at this dose.

Conclusions

Strengths

This is a cell-based therapy trial for COVID-19 based on a third party of NK cells obtained from convalescent donors that contains the memory SARS-CoV-2 NK cell phenotype, $CD57^{+}$ $NKG2C^{+}$. This NK cell subset may be crucial for the specific response against SARS-CoV-2. This is a unique approach selecting the best donors, if we compare with other nonspecific SARS-CoV-2 NK cell products such as a cord blood-derived allogeneic NK cell therapy.[31] It guarantees a universal and quickly accessible product whenever needed. The manufacturing method is homogeneous, and all patients were infused from the same product obtained from one healthy donor. The access to NK cell therapy may also be anticipated, typically requiring standard hematological medical infrastructure. Furthermore, this trial has been run by a team that includes clinical doctors that treat patients and manufacturing health care providers in the same facilities. This situation avoids partial disconnection between clinical centers versus laboratories that manufacture cell therapy products. Hopefully, the testing of NK cell therapies will be pursued synergically in parallel to the improvement of standard care.

Limitations of the study

We are aware of the main limitations of the study. The patient group is small, highlighting the difficulty to accrue patients for clinical trials that treat severe symptoms of COVID-19 infection. For this reason, our statistical analysis is merely descriptive. Hence, we cannot assume RP2D or efficacy. There may never be enough data to fully explore the efficacy of cell-based therapy against COVID-19. Furthermore, the relative impact of immune cells may never be completely understood within the current setting of COVID-19, being vaccination the most valuable tool available to prevent and lessen severe cases. However, we will still need treatment for severe vaccinated or unvaccinated patients. In addition, in comparison with third party memory T cells, the cost is also slightly higher. Although we observed the feasibility and safety of third-party NK cells containing the memory SARS-CoV-2 NK cell phenotype, more research is needed to address which group of patients may benefit.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Antonio Pérez Martínez (aperezmartinez@salud.madrid.org).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conception and design of the study: A.P.-M., C.E., C.H.-B., K.A.-S., and L.H. Acquisition of data: C.H.-B., K.A.-S, L.H., C.E., and A.P.-M. Analysis and interpretation of data: C.H.-B., K.A.-S., L.H., D.L.-O., L.P.-R., J.C.-O., P.G.-G., A.M.-Q., J.L.V., S.S., M.A.P.-V., M.A.V., A.M.M.B., A.J.J.C. A.B., M.A.M., R.P.d.D., M.G., B.S., C.E., and A.P.-M. Drafting or revising the manuscript: C.H.-B., K.A.-S., L.H., C.A.-P., C.E., and A.P.-M. All authors have approved the final article.

DECLARATION OF INTERESTS

The authors have no conflicts of interest to declare.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
7AAD	Miltenyi Biotec	Cat#130-111-568
Anti-CD158a PE	BD Pharmigen	RRID: AB_396332
Anti-CD158b FITC	BD Pharmigen	RRID: AB_397325
Anti-CD158e APC	BD Pharmigen	RRID: AB_2738594
Anti-CD16 BV421	BD Pharmigen	RRID: AB_2716865
Anti-CD3 FITC	BD Pharmigen	RRID: AB_2869864
Anti-CD3 PerCPCy5.5	Biolegend	RRID: AB_10640736
Anti-CD56 FITC	BD Pharmigen	RRID: AB_2737799
Anti-CD56 PECy7	Biolegend	RRID: AB_2149542
Anti-CD56 APC	Miltenyi Biotec	RRID: AB_2726088
Anti-CD57 PE	BD Pharmigen	RRID: AB_2033965
Anti-CD57 APC	BD Pharmigen	RRID: AB_10563760
Anti-CD96 BV421	BD OptiBuild	RRID: AB_2741051
Anti-CTLA4 FITC	Invitrogen	RRID: AB_2637119
Anti-DNAM-1 BV510	BD OptiBuild	RRID: AB_2740827
Anti-EOMES PE	BD Pharmigen	RRID: AB_2869847
Anti-Granzyme B FITC	BD Pharmigen	RRID: AB_1645488
Anti-IFN γ APC	BD Pharmigen	RRID: AB_10893191
Anti-NKG2A FITC	Miltenyi Biotec	RRID: AB_2733623
Anti-NKG2A BV510	BD OptiBuild	RRID: AB_2872383
Anti-NKG2C BV510	BD OptiBuild	RRID: AB_2872628
Anti-NKG2D APC	BD Pharmigen	RRID: AB_398654
Anti-NKp30 PE	BD Pharmigen	RRID: AB_647240
Anti-NKp44 PE	Miltenyi Biotec	RRID: AB_2752113
Anti-NKp46 BV510	BD Horizon	RRID: AB_2738571
Anti-PD-1 BV510	BD Horizon	RRID: AB_2737990
Anti-Perforin PE-Vio-770	Miltenyi Biotec	RRID: AB_2922353
Anti-T-bet BV421	BD Horizon	RRID: AB_2687543
Anti-TIGIT PE	Invitrogen	RRID: AB_10714831
Anti-TIM3 APC	Invitrogen	RRID: AB_1963622
Anti-CD3 Viogreen	Miltenyi Biotec	RRID: AB_2725970
Anti-CD56 AF700	BD Pharmingen	RRID: AB_396940
Anti-CD25 BV421	BD Horizon	RRID: AB_2738555
Anti-HLA-DR BV421	BD Horizon	RRID: AB_2687421
Anti-CD69 PE	Miltenyi Biotec	RRID: AB_2659065
Chemicals, peptides, and recombinant proteins		
PepTivator® SARS-CoV-2 Prot_S1, research grade	Miltenyi Biotec	130-127-041
PepTivator® SARS-CoV-2 Prot_M, research grade	Miltenyi Biotec	130-126-703
PepTivator® SARS-CoV-2 Prot_N, research grade	Miltenyi Biotec	130-126-698
Critical commercial assays		
Human IFN-γ Secretion Assay-Detection Kit	Miltenyi Biotec	130-090-433
LEGENDPlexTM human cytokine panel kit	BioLegend	741027
Foxp3/Transcription Factor Staining Buffer Set	Invitrogen	00-5523-00

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
FlowJo 10.7.1 software	BD Company	
LEGENDplex™ Data Analysis Software Suite	BioLegend	
GraphPad Software		

EXPERIMENTAL MODEL AND STUDY PARTICIPANT

Study design and study participant details

RELEASE phase I clinical trial is an open-label, dose-finding clinical trial to evaluate the safety of the administration of NK cells in COVID-19 patients with lymphopenia and/or pneumonia. It was conducted at La Paz University Hospital (La Paz), Madrid, Spain, between January and July 2022. The trial was approved by the institutional review board and was conducted following good clinical practice guidelines and the Declaration of Helsinki. All patients provided written informed consent. The authors vouch for the accuracy and completeness of the data and the fidelity of the trial to the protocol. The protocol of the trial was approved by the Ethics Committee of La Paz University Hospital (identifier: Clinical Ethical Approval No. HULP: 5579) and overseen by a data and safety monitoring board. All of the authors participated in writing the submitted manuscript (Clinicaltrials.gov registration: NCT04578210). Participants were adults of Caucasian origin, with mean age of 63 years, five male and one female. For detailed characteristics of the patients, see Table 1.

METHOD DETAILS

Inclusion criteria and monitoring

Patients aged between 18 and 80 years were screened for enrollment from the emergency department at admission to La Paz, with a positive polymerase chain reaction (PCR) assay or antigen test for SARS-CoV-2, radiologically confirmed pneumonia and/or lymphopenia, baseline oxygen saturation $\leq$94% on room air, and less than 12 days since symptom onset. Clinical and analytical monitoring was performed (Table S3). After providing their informed consent, six participants were enrolled in the trial. The trial was terminated without completing the expected recruitment because global COVID-19 vaccination resulted in less severe clinical presentations. Nevertheless, given the difficulties in recruiting patients for cell therapy trials, we consider it important to report the outcomes of these 6 patients.

Intervention

Eligible participants were studied for human leukocyte antigen (HLA) class I and KIR. Participants were sequentially enrolled to receive a single infusion in a dose-escalating manner. Given that our provision of NK cells was obtained from 2 donors, the doses of the original RELEASE trial were modified, with a calculated progressive dose of 1×10^6 cells/kg (cohort 1), 2×10^6 cells/kg (cohort 2), and 3×10^6 cells/kg (cohort 3). All patients received standard of care treatment according to the international guidelines for COVID-19 at the time. All concomitant medication was recorded, from the day the informed consent was signed until the patient was discharged.

The participants were infused with $CD3^-/CD56^+$ NK cells through a standard blood filter in a gravity-driven system previously medicated with intravenous diphenhydramine 5 mg and acetaminophen 1 mg.

Donor selection and procedure for memory natural killer cell selection and preservation

Two donors were selected based on the presence of SARS-CoV-2-specific memory NK cells ($CD57^+/NKG2C^+$) and the time since COVID-19 infection (within 4 months post-infection). Selection was conducted in 2020 prior to the Spanish vaccination campaign; both donors were therefore unvaccinated convalescent patients. The percentage of $CD3^-$ $CD56^+$ cells was 99% and 93% post-$CD3^+$ depletion and $CD56^+$ selection, respectively, and the total cell number was 9.31×10^8 and 5.77×10^8, respectively, although only one donor was ultimately used for infusion.

Blood donor selection was performed by the Basque Center for Blood Transfusion and Human Tissues, according to the following criteria: $\leq$65 years of age, positive SARS-CoV-2 test by PCR, and complete resolution of symptoms for at least 14 days before donation. The donor was required to have at least one SARS-CoV-2-negative test by PCR from a nasopharyngeal swab or, if available, a negative SARS-CoV-2 viremia tested by quantitative PCR before donation. Donor's blood was HLA and KIR typed, and donor's characteristics and aphaeresis were obtained. As described in previous preclinical research, we determined the $CD57^+/NKG2C^+$ phenotype NK cells after COVID-19 in convalescent donors by non-mobilized aphaeresis and NK- cell product enrichment.[9] Briefly, non-mobilized aphaeresis was performed at the Basque Center for Blood Transfusion and Human Tissues (Galdakao, Spain), and the NK-cell product enrichment was performed at the Bone Marrow Transplantation and Cell Therapy Unit at La Paz using a CliniMACS Plus cell separation system (Miltenyi Biotec).[8]

Following aphaeresis, $CD56^+$ cells were purified according to a 2-step protocol: first, the CD3 population was depleted using CD3 reagent and a depletion program. Next, the $CD56^+$ population was enriched by employing clinical-grade CD56 microbeads and an enrichment program in the CliniMACS device. $CD3^-/CD56^+$ cells were aliquoted for cryopreservation in doses adjusted to 100 kg of weight and the 3 planned doses. Aliquots were cryopreserved in a double bag using 50% PlasmaLyte, 40% donor autologous plasma, and a final concentration of 10% dimethyl sulfoxide (DMSO). $CD3^-/CD56^+$ aliquots were removed from liquid nitrogen storage and thawed in a dry defroster. Infusion of cryopreserved $CD3^-/CD56^+$ lymphocytes was performed 15 min after thawing. $CD3^-/CD56^+$ fraction viability and purity were analyzed by flow cytometry and are shown in Table S4.

Purified NK cells from donor 1 and donor 2 were thawed to perform functional assays and immunophenotype analysis. For the functional assays, NK cells were stimulated with individual and pooled SARS-CoV-2 peptides (M, N, S) (Miltenyi), as previously described in Herrera et al.[9] NK cells were checked for the presence of $CD57^+$ $NKG2C^+$ double-positive population, the so-called memory NK cells, as well as other activating and inhibitory markers such as NKG2A,[32] CD96, NKp30, NKp44, NKp46 and NKG2D. CD16 marker expression level was analyzed to determine the cytotoxic NK cell subpopulation (Figure S4).

Immune human leukocyte antigen and killer cell immunoglobulin-like receptor typing

The HLA and KIR genotyping of the convalescent donor was performed at the Basque Center for Blood Transfusion and Human Tissues (Basque Country, Spain) by NGS and Luminex, respectively. The chosen donor genotype was the following: HLA:A*01:01P, A*01:01P/B*44:05P, B*55:01P/C*01:02P, C*02:02P/DRB1*07:01P, DRB1*13:01P/DQB1*03:03P, DQB1*06:03/DPB1*04:01P, DPB1*14:01P. KIR: KIR2DL1, KIR2DL2, KIR2DL3, KIR2DL4, KIR2DP1, KIR2DS2, KIR2DS4, KIR3DL1, KIR3DL2, KIR3DL3, KIR3DP1; haplotype AB/AA.[8] HLA genotyping of enrolled patients was performed at the Transfusion Center of the Community of Madrid.

Study procedures

The study visits and the procedures performed are shown in Table S3. Study participants who met the eligibility requirements, fulfilling all inclusion criteria and none of the exclusion criteria, were immediately asked to participate. Inclusion and dosing occurred on the same day if possible. Participants must have had completed the following assessments before infusion: physical examination, respiratory status, blood sample collection (on day 1 for hematological, biochemical, and immunological function markers, and donor chimerism analysis), and a record of any AE. These assessments, including a weekly PCR check, were repeated on the subsequent study visits. Additional assessments were performed when possible. Patients were followed-up weekly until day 28, and at one year, or until death, had it occurred.

Outcomes

The primary outcome was to determine the safety of a single infusion of NK cells from a healthy donor who recovered from COVID-19. The aim was to determine the RP2D and DLT for a subsequent phase II clinical trial. DLT is defined as any grade 3 or higher AE directly or probably related to the cell infusion and any lower grade AE that increases to a grade 3 or higher as a direct result of the cell infusion, up to 21 days after cell infusion. AEs were assessed and graded according to the Common Terminology Criteria for Adverse Events version 5.0.

Secondary outcomes were to evaluate time to lymphopenia recovery and to immune dysregulation, the time needed for a negative SARS-CoV-2 result by PCR, clinical improvement through the NEWS and a 7-category point ordinal scale,[14,15] and length of stay. Laboratory findings of inflammatory markers were monitored as well.

Exploratory outcomes included the monitoring of donor chimerism, NK-cell immunophenotype evolution, and immune lymphocyte reconstitution during the 3 months following the infusion and immunological profiling of patients. NK function in the presence of corticoids was also monitored.

Chimerism

Micro-chimerism was defined as the percentage of NK cells of the donor that are detected by PCR in the patient after infusion, allowing us to detect that the donor cells are alive and viable after infusion. For each donor-recipient pair, DNA was isolated using the QIAamp Blood Kit (Qiagen, Hilden, Germany). Chimerism was monitored weekly for 3–4 consecutive weeks and was analyzed based on the detection of insertion/deletion polymorphism by quantitative PCR technology (sensitivity 0.01%–0.05%). Commercial reagents for screening of informative alleles (Mentype DIPscreen, Biotype, Dresden, Germany) and quantitative chimerism analysis (Mentype DIPquant qPCR, Biotype) were employed. The percentage of donor alleles was calculated based on the ddCt quantitative PCR method by using Chimerism Monitor 2.1 software (Biotype), b-globin being the reference gene.

Patients' natural killer cell immunophenotype

Immunophenotyping of peripheral blood $CD3^+$ T cell subsets ($CD4^+$ and $CD8^+$ naive/memory $CD45RA^+/CD45RO^+$, NKT $CD3^+CD16^+CD56^+$, regulatory T cells $CD4^+CD25^+CD127^{low}$ and activated T cells $CD3^+HLADR^+$), NK cells ($CD3^-CD16^+CD56^+$), and B cells ($CD19^+$) was performed by multiparametric flow cytometry. Peripheral blood was collected in ethylenediaminetetraacetic acid tubes, and cells were stained for various surface markers with fluorochrome-conjugated anti-human antibodies (Beckman Coulter BC, Indianapolis, IN, USA): anti-CD45RO FITC, anti-CD27 PE, anti-CD3 ECD, anti-CD19 PC7, anti-TCR$\gamma\delta$ PC7, anti-CD16

APC, anti-CD56 APC, anti-CD8 APC AF700, anti-CD4 APC AF750, anti-CD45 KO, anti-CD25 FITC, anti-CD31 PE, anti-CD45RA PC7, anti-CD127 APC, and anti-HLA-DR PB. Cells were acquired by flow cytometry on a DxFlex cytometer and analyzed with Kaluza Software (Beckman Coulter BC, Indianapolis, IN, USA).

For the immunophenotype of NK cells, blood samples were obtained from the participants at various time points after infusion (days 0, 3, 7, 14, 21, and 28). Peripheral blood mononuclear cells (PBMCs) were cryopreserved in a double bag using 50% PlasmaLyte, 40% donor autologous plasma, and a final concentration of 10% DMSO. When performing the immunophenotype, PBMCs were thawed and subsequently preserved at 37°C for 1 h. Cells were subsequently washed twice with phosphate-buffered saline plus 10% fetal bovine serum and centrifuged at 300 g, at 4°C for 5 min. Dead cells were identified by staining them for 30 min on ice with LIVE/DEAD Fixable Near-IR. Extracellular markers were stained for 30 min on ice. Cells were then fixed and permeabilized with a Fox3/Transcription Factor Staining Buffer Set (eBiocience, Thermo Fisher Scientific) following the manufacturer's recommendations. Lastly, cells were stained with intracellular markers for 30 min on ice. Cell acquisition was performed with a BD FACSCANTO II, acquiring an average of 100,000 cells. All the antibody markers employed in this process are listed in key resources table. The analysis was performed using FlowJo 10.6.3 (FlowJo, LLC). The gating strategy for T cell and NK cell analysis can be find at Figure S5.

Quantification of circulating levels of pro-inflammatory cytokines

Serum samples were collected from six patients at six separated time points (pre-infusion [day 0] and days 3, 7, 14, 21, and 28 post-infusion) and peripheral pro-inflammatory cytokines (interleukin [IL]-5, IL-13, IL-2, IL-6, IL-9, IL-10, IL-17A, IL-17F, IL-4, IL-22, Interferon-gamma [IFN-γ], and tumor necrosis factor-alpha [TNF- α]) were quantified by using a LEGENDPlex human cytokine panel kit (BioLegend) following manufacturer's instructions. Acquisition was carried out in a 5-laser Cytek Aurora device (Cytek Biosciences) and the data were analyzed using the LEGENDplex Data Analysis Software Suite (BioLegend).

Natural killer function and phenotype in the presence of dexamethasone

Cellular NK response against SARS-CoV-2 was evaluated by measuring the capacity of IFN-γ production, in the presence of various concentrations of dexamethasone (no dexamethasone, 10^{-7} M, 10^{-6} M, 10^{-5} M), with the dose of 10^{-7} M corresponding to 6 mg dexamethasone, employed in standard clinical practice. The activation state of the cells was evaluated as well by staining cells with the activation markers HLA-DR, CD69, and CD25. The assay was performed as previously described by our group.[33] Briefly, PBMCs were stimulated with a combination of SARS-CoV-2 peptide pools (M, N, S) (Miltenyi Biotec) and labeled with interferon-gamma (IFN-γ) catch reagent Kit (Miltenyi Biotec) and detected by Flow Cytometry. Background subtraction was performed from parallel unstimulated cultures. A positive sample included the following acceptance criteria: 0.1% of IFN-γ+ cells out of the total cell population with a minimum of 150,000 events analyzed, at least twice the number of IFN-γ+ cells in the sample than in the negative control and a positive control based on plate-bound cells stimulated with mouse anti-human CD3 and co-stimulated with purified CD28/CD49d. After incubation, cells where stained using the following fluorochrome-conjugated anti-human surface antibodies: CD3 Viogreen, CD56 AF700, L/D 7AAD and IFN-γ PE. Cell acquisition was then performed using a Navios cytometer (Beckman Coulter, Indianapolis, IN, USA), acquiring a mean of 200,000 cells, and the analysis was performed using FlowJo 10.7.1 software. Phenotype of NK cells was determined by flow cytometry using the following fluorochrome-conjugated antihuman antibodies: CD3 Viogreen, CD56 AF700, L/D 7AAD, CD25 BV421, HLA-DR BV421 and CD69 PE.

Data management and study monitoring

Categorical data are presented as numbers and percentages when applicable. Descriptive tables and figures are employed to enable data visualization. An electronic case report form (eCRF; Appendix 2) was designed with MACRO Electronic Data Capture by Elsevier and included all the variables described. This system anonymizes patients and the data were analyzed with R software (V.3.5.2 or newer). Data management and statistical plans were approved by the principal investigator and the sponsor. Data collection forms will be included in the final report. Data management was performed by the Spanish Clinical Research Network (SCReN). Trial coordination, management, monitoring, and statistical analysis were performed by the Clinical Trial Unit (Department of Clinical Pharmacology and IdiPAZ) of La Paz, belonging to the SCReN.

Role of the funding source

The study sponsor had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all of the study data and had ultimate responsibility for the decision to submit for publication.

QUANTIFICATION AND STATISTICAL ANALYSIS

To check the effects of different concentrations of dexamethasone on NK cells, two-way ANOVA tests with Turkey's multiple comparisons were performed in all cases: to observe changes in the IFN-γ production, in the activation phenotype or in the total numbers

of NK cells. N varies between four and seven depending on the number of experiments performed, and the graphics represent in all cases mean and standard error of the mean (SEM) of the values obtained. Any *p* value lower than 0.05 was considered statistically significant.

ADDITIONAL RESOURCES

RELEASE Clinical Trial is registered at Clinicaltrials.gov registration: NCT04578210.