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Allogeneic, off-the-shelf, SARS-CoV-2-specific T cells (ALVR109) for the treatment of COVID-19 in high-risk patients

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Supplementary Materials

Supplementary Methods

Flow Cytometry

Surface Immunophenotyping

SARS-CoV-2-VSTs were surface-stained with monoclonal antibodies to: CD3, CD28, CD69, CD45RO, CD279 (PD-1) [Becton Dickinson (BD), Franklin Lakes, NJ], CD4, CD8, CD16, CD62L (Beckman Coulter, Brea, CA), CD56 and CD366 (TIM-3) (BioLegend, San Diego, CA). Cells were pelleted in phosphate-buffered saline (PBS) (Sigma-Aldrich), then antibodies added in saturating amounts (5µl) followed by incubation for 15mins at 4°C. Subsequently, cells were washed, resuspended in 300µl of PBS and at least 20,000 live cells acquired on a Gallios™ Flow Cytometer and analyzed with Kaluza® Flow Analysis Software (Beckman Coulter).

Intracellular Cytokine Staining (ICS)

SARS-CoV-2-VSTs were harvested, resuspended in VST medium ($2x10^6$/ml) and 200µl added per well of a 96-well plate. Cells were incubated overnight with 200ng of individual test or control pepmixes along with Brefeldin A (1µg/ml), monensin (1µg/ml), CD28 and CD49d (1µg/ml) (BD). Next, VSTs were washed with PBS, pelleted, surface-stained with CD8 and CD3 (5µl/antibody/tube) for 15mins at 4°C, then washed, pelleted, fixed and permeabilized with Cytofix/ Cytoperm solution (BD) for 20mins at 4°C in the dark. After washing with Perm/Wash Buffer (BD), cells were incubated with 10µl of IFNγ and TNFα antibodies (BD) for 30min at 4°C in

the dark. Cells were then washed twice with Perm/Wash Buffer and at least 50,000 live cells were acquired on a Gallios™ Flow Cytometer and analyzed with Kaluza® Flow Analysis Software.

TCR vβ immunophenotyping

TCRvβ flow cytometric analysis was performed using the IOTest® Beta Mark kit (Beckman Coulter, Brea, CA), per manufacturer's instructions. Briefly, $0.5x10^6$ cells per tube were surface-stained with CD3 and the TCRvβ-specific monoclonal antibodies provided with the kit and incubated for 20 minutes at room temperature in the dark. Subsequently, cells were washed, resuspended in 300µl of PBS and at least 10,000 live T cells acquired on a Gallios™ Flow Cytometer and analyzed with Kaluza® Flow Analysis Software (Beckman Coulter).

Functional Studies

Enzyme-Linked Immunospot (ELISpot)

ELISpot analysis was used to quantitate the frequency of IFNγ and Granzyme B-secreting cells. Briefly, PBMCs and/or SARS-CoV-2-VSTs were resuspended at $5x10^6$ or $2x10^6$ cells/ml in VST medium and 100µl of cells was added to each ELISpot well. Antigen-specific activity was measured after direct stimulation (500ng/peptide/ml) with the individual stimulating or control pepmixes (Survivin, WT1). Staphylococcal Enterotoxin B (SEB) (1µg/ml) and PHA (1µg/ml) were used as positive controls for PBMCs and VSTs, respectively. After 16-18 hours of incubation, plates were developed as previously described, dried overnight at room temperature and then quantified using the IRIS ELISpot/FluoroSpot reader (Mabtech, Inc., Cincinnati, OH). Spot-forming cells (SFC) and input cell numbers were plotted and the specificity threshold for VSTs was defined as ≥30 SFC/$2x10^5$ input cells.

FluoroSpot

For the quantitation of polyfunctional cells simultaneously secreting IFNγ, Granzyme B and/or TNFα a commercial FluoroSpot assay was used (Human IFNγ/Granzyme B/TNFα FluoroSpot Plus, Mabtech, Inc., Cincinnati, OH). Briefly, SARS-CoV-2-VSTs were resuspended at 0.5 to $2x10^6$ cells/ml in VST medium and 100µl of cells were added to each FluoroSpot well. Antigen-specific activity was measured after direct stimulation (500ng/peptide/ml) with the individual stimulating or control pepmixes. After a minimum of 18 hours of incubation, plates were developed as per manufacturer's instructions and then visualized and quantified using the IRIS ELISpot/FluoroSpot reader.

Multiplex

The SARS-CoV-2-VST cytokine profile was evaluated using the MILLIPLEX High Sensitivity Human Cytokine Panel (Millipore, Billerica, MA). $2x10^5$ VSTs were stimulated with SARS-CoV-2 pepmixes (1µg/ml) overnight. Subsequently, supernatant was collected, plated in duplicate wells, incubated overnight at 4°C with antibody-immobilized beads, then washed and plated for 1 hour at room temperature with biotinylated detection antibodies. Finally, streptavidin-phycoerythrin was added for 30 minutes at room temperature. Samples were washed and analyzed on a Luminex 200 (XMAP Technology) using the xPONENT software.

Chromium release assay

A standard 4-6 hour chromium (Cr^{51}) release assay was used to measure the specific cytolytic activity of SARS-CoV-2-VSTs with autologous antigen-loaded PHA blasts as targets (20ng/pepmix/$1x10^6$ target cells). Effector:Target (E:T) ratios of 80:1, 40:1, 20:1, 10:1, and 5:1 were used to analyze specific lysis. The percentage of specific isotope release was calculated [(experimental release - spontaneous release)/(maximum release - spontaneous release)] x 100.

In order to measure the autoreactive and alloreactive potential of SARS-CoV-2-VST lines, autologous and allogeneic PHA blasts alone were used as target cells.

Clinical Trial

Patients hospitalized with COVID-19 (PCR proven), and with at least 2 CDC-defined risk factors for progression to severe COVID-19 disease were eligible to participate in a protocol that was conducted under an FDA-cleared IND with BCM IRB approval (H-47739, NCT 04401410). Key risk factors were: age ≥60 years, obesity (BMI≥30), post-HSCT or solid organ transplantation, diabetes, and cancer diagnosis on active treatment (within 3 months of last therapy). Once enrolled, patients received a single infusion of a partially HLA-matched (at least 2/8 antigen match) SARS-CoV-2-specific T cell line (ALVR109) on either dose level (DL) 1 or 2 (1×10^7 or 2×10^7 cells). While hospitalized, all patients underwent daily assessment for signs or symptoms of acute graft-versus-host-disease (GVHD), cytokine release syndrome (CRS) or immune effector cell associated neurotoxicity (ICANS). Post-discharge, patients were evaluated at week 2 post-infusion, as well as months 2, 3 and 6. Development of grade ≥III GVHD, CRS or ICANS or failure of grade II toxicities to respond to standard measures were defined as dose limiting toxicities (DLTs). Accrual to the pilot (first) phase of the study began on 11/1/2020 and the last follow-up date on study is 10/15/2021. A full list of inclusion/exclusion criteria as well as a detailed schedule of clinical assessments is supplied in the submitted clinical protocol (Supplementary Materials).

As originally designed, this study was to have two parts as outlined in the clinical protocol. First, patients with COVID-19 who were at high risk of progression to mechanical ventilation were to be enrolled in a dose-finding phase in order to identify the maximum tolerated dose (MTD). Next, a randomized trial using the ALVR109 MTD was to be conducted, with approximately 40 patients randomized 1:1 to receive ALVR109 or routine treatment per institutional standards. When the

protocol was originally approved in May of 2020, the population of hospitalized COVID-19 was high and the expectation that enrollment goals could be quickly reached seemed reasonable. However, few hospitalized COVID-19 patients met the eligibility criteria for the study and as the population of hospitalized patients declined and COVID-19 treatments were provisionally approved, full enrollment in a reasonable time frame was no longer feasible. Eventually, we decided to halt the trial after four patients were enrolled and dosed.

VST in vivo persistence studies

To track the infused VSTs, high-throughput deep sequencing of TCRvβ CDR3 regions was applied (Adaptive Biotechnologies, Seattle, WA). Deep sequencing was performed on the infused lines and on peripheral blood samples collected before and after infusion. Those T cell clones identified within the product but not detected in patients' pre-infusion repertoire were coded as line-derived unique clones.

Statistical Analysis

Descriptive statistics were calculated to summarize preclinical data and clinical characteristics. Dose escalation was performed using the 3+3 design (see protocol in Supplementary Materials) to determine the maximum tolerated dose (MTD) of VSTs, with MTD defined as the highest DL at which the probability of a DLT was ≤33%. In this paper, partial trial results are presented to demonstrate feasibility and lack of toxicity.

Supplementary Tables

Supplementary Table 1. SARS-CoV-2 antigen hierarchy of immunodominance

PBMCs

Antigens	# responding donors	SFC/5x10^5 PBMCs (mean±SEM)
S	16	197±40
E	1	6±2
M	14	90±19
N	15	109±25
Nsp1	1	4±1
Nsp3	3	8±2
Nsp4	3	8±2
Nsp5	1	4±1
Nsp6	1	5±1
AP7A	5	8±2
AP7B	0	2±1
AP8	2	6±1
Nsp10	1	4±1
Nsp12	1	4±1
Nsp13	1	6±2
Nsp14	0	2±1
Nsp15	0	2±1
Nsp16	1	6±2

VSTs

Antigens	# responding donors	SFC/2x10e5 VSTs (mean±SEM)
S	**16**	**4700 ± 880**
N	**16**	**2568±547**
M	**16**	**2300±454**
AP7a	**10**	**280±133**
Nsp4	**9**	**450±282**
AP8	8	113±43
E	7	53±15
Nsp6	7	43±14
Nsp16	5	58±42
Nsp3	5	54±32
Nsp5	5	27±8
Nsp13	4	31±15
Nsp12	4	23±7
AP7b	3	32±19
Nsp1	3	21±8
Nsp14	2	19±8
Nsp10	2	14±6
Nsp15	2	14±6

Supplementary Table 2. VST line characteristics

VST No.	HLA Type				Specificity (SFC/2x10^5)					Phenotype (% expression)						Alloreactivity (20:1)
	A	B	DRB1	DQB1	S	M	N	AP7a	Nsp4	CD3	CD4	CD8	CD3-CD56+	Tcm	Tem	% specific lysis
9587.41.BAT	02, 68	35, 39	04, 08	03, 04	416	252	589	2	31	99.85	73.60	23.21	0.11	36.47	59.23	-1.08
9489.42.BAT	02, 03	15, 38	07, 15	02, 06	792	18	221	0	6	99.64	91.18	7.33	0.06	49.82	45.88	-0.9
9526.41.BAT	02, 25	18, 40	11, 15	03, 06	1447	941	376	8	59	99.71	90.40	5.55	0.05	59.15	35.61	-0.9
9778.41.BAT	11, 29	35, 44	07, 14	02, 05	3085	582	2613	24	1	99.52	83.23	13.17	0.09	63.20	31.96	1.1
9526.42.BAT	02, 25	18, 40	11, 15	03, 06	568	198	295	0	4	99.80	97.13	1.44	0.06	49.20	48.99	-1.85
9479.41.BAT	02, 68	44, 51	04, 07	02, 03	447	388	225	0	0	99.56	96.38	2.06	0.09	48.52	49.10	1.06
9479.42.BAT	02, 68	44, 51	04, 07	02, 03	535	467	315	0	0	99.60	94.68	3.10	0.09	34.09	62.44	-1.84
9479.43.BAT	02, 68	44, 51	04, 07	02, 03	377	487	1341	20	1	99.13	95.58	2.01	0.42	66.05	30.79	-0.28
9489.41.BAT	02, 03	15, 38	07, 15	02, 06	640	20	151	0	10	99.61	78.30	14.95	0.46	34.81	51.54	-0.19
9480.42.BAT	24, 24	07, 15	12, 14	02, 03	1195	117	363	426	0	99.25	95.59	2.11	0.02	67.41	29.72	0.85
9587.42.BAT	02, 68	35, 39	04, 08	03, 04	1967	988	1626	256	257	99.61	90.10	7.19	0.12	42.68	54.31	-1.95
9775.41.BAT	02, 03	15, 44	04, 12	03, 03	1043	3	240	0	0	98.97	85.99	11.42	0.12	62.42	35.05	1.37
9778.42.BAT	11, 29	35, 44	07, 14	02, 05	1142	578	1193	18	0	99.73	84.84	8.68	0.08	70.53	22.40	0.2
9837.41.BAT	24, 24	40, 45	10, 16	03, 05	2135	1368	1000	35	0	94.53	85.97	4.80	0.58	20.04	69.72	0.13
9837.42.BAT	24, 24	40, 45	10, 16	03, 05	3672	1145	1244	13	3	95.24	86.26	2.62	0.38	41.85	45.89	-5.84

Supplementary Table 3. HLA matching between patients and infused lines

Pt HLA					Infused VST line HLA				
Pt ID	**A**	**B**	**DR**	**DQ**	**A**	**B**	**DR**	**DQ**	**VST No.**
Pt1	02, 02	39, 40	04, 08	04, 06	02, 68	35, 39	04, 08	03, 04	C9587.41.BAT
Pt2	01, 02	08, 38	03, 13	02, 06	02, 03	15, 38	07, 15	02, 06	C9489.42.BAT
Pt3	11, 32	52, 57	15, 15	06, 06	02, 25	18, 40	11, 15	03, 06	C9526.41.BAT
Pt4	03, 11	27, 35	04, 15	06, 03	11, 29	35, 44	07, 14	02, 05	C9778.41.BAT

Supplementary Table 4. Treatment-related AEs

DL	Incident	# of patients	Onset in relation to infusion	Max grade	Treatment, if any	Status
DL1	Skin rash	1	D+13	2	Resumption of dexamethasone at doses useful in COVID19 patients (6)	Resolved 2 days later
DL2	CRS (Fever and hypoxia)	1	D+3 (Grade II) progressed on D+13 (Grade III)	3	Supportive measures and 2 week course of corticosteroids	Stabilized over the first 2 weeks then was gradually weaned off O_2 supplementation within 2 months.

AE: adverse event, DL: dose level, Max: Maximum grade seen in any of the patients at that dose level, CRS: Cytokine release syndrome, O_2: oxygen

Supplementary Table 5. VST line-derived TCR clonotypes detected in treated patients

Pt#	CDR3 nucleotide sequence	TCR Bioidentity	Aminoacids	ORF coverage	HLA restriction	Detection				
1	AATCTTCACATCAATTCCCTGGAGCTTGGTGACTCTGCTGTGTATTTCTGTGCCAGCAGCCAAGCCGGGGAGCTGTTTTTTGGAGAA	CASSQAGELFF+TCRBV03-01/03-02+TCRBJ02-02	FNDGVYFASTEKSNIIRGW,LFLPFFSNVTWFHAIHVSG,RFDNPVLPFNDGVYFASTE,STEKSNIIRGWIFGTTLDS,VSGTNGTKRFDNPVLPFND,VTWFHAIHVSGTNGTKRFD	Spike	A*02	D1	Wk2	Mo2		
2	TTAAATCTTCACATCAATTCCCTGGAGCTTGGTGACTCTGCTGTGTATTTCTGTGCCAGCAGCCAAGAGACCCAGTACTTCGGGCCA	CASSQETQYF+TCRBV03-01/03-02+TCRBJ02-05	AFLLFLVLI,FLAFLLFLV,FYLCFLAFL,FYLCFLAFLL,IDFYLCFLAF,IELSLIDFYL,LIDFYLCFL,LLFLVLIML,MIELSLIDFY,SLIDFYLCFL,YLCFLAFLL	ORF7a/7b	A*02	D1	Wk2	Mo2		
	AGGATCCAGCAGGTAGTGCGAGGAGATTCGGCAGCTTATTTCTGTGCCAGCTCACCAGGGGGGGGCACTGAAGCTTTCTTTGGACAA	CASSPGGGTEAFF+TCRBV18-01+TCRBJ01-01	DTDFVNEFYAY,NRDVDTDFVNEFY	ORF1ab	A*02	D1	Wk2	Mo2		
3	AATCTTCACATCAATTCCCTGGAGCTTGGTGACTCTGCTGTGTATTTCTGTGCCAGCAGCCAAGCCGGGGAGCTGTTTTTTGGAGAA	CASSQAGELFF+TCRBV03-01/03-02+TCRBJ02-02	FNDGVYFASTEKSNIIRGW,LFLPFFSNVTWFHAIHVSG,RFDNPVLPFNDGVYFASTE,STEKSNIIRGWIFGTTLDS,VSGTNGTKRFDNPVLPFND,VTWFHAIHVSGTNGTKRFD	Spike	DR*15, DQ*06	D1		Wk2		
	AAGATCCAGCCCTCAGAACCCAGGGACTCAGCTGTGTACTTCTGTGCCAGCAGTTTCGGGGGGGAACTATGGCTACACCTTCGGTTCG	CASSFGGNYGYTF+TCRBV12-03/12-04+TCRBJ01-02	GAGAALQIPFAMQMAYRFN,GLTVLPPLLTDEMIAQYTS,LICAQKFNGLTVLPPLLTD,LTDEMIAQYTSALLAGTIT,TITSGWTFGAGAALQIPFA,YTSALLAGTITSGWTFGAG	Spike	DR*15, DQ*06	D1		Wk2		
4	GTGACATCGGCCCAAAAGAACCCGACAGCTTTCTATCTCTGTGCCAGTGGGACAGGGGATAGCAATCAGCCCCAGCATTTTGGTGAT	CASGTGDSNQPQHF+TCRBV19-01+TCRBJ01-05	HTTDPSFLGRY	ORF1ab	not known	D1	Wk2	Mo3	Mo4	Mo6

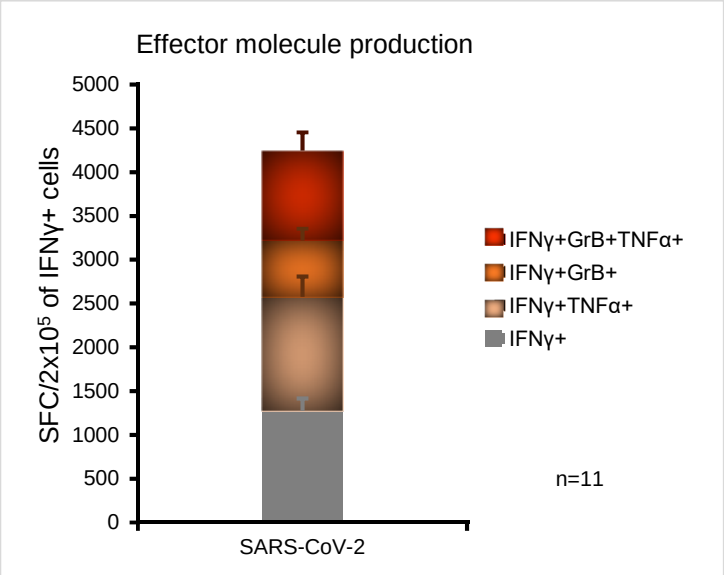
Detected Not detected

Supplementary Figure Legends

Supplementary Figure 1: Polyfunctionality of ex vivo expanded SARS-CoV-2 VSTs. Quantitative assessment of single (IFNγ+), dual (IFNγ+TNFα+/IFNγ+GrB+) and triple (IFNγ+TNFα+GrB+) effector molecule-producing cells as measured by FluoroSpot. Data is reported as SFC ± SEM.

Supplementary Figure 2: Amino acid sequence of the parental Spike protein. The positions of selected mutations are highlighted in blue. Unique Immunogenic Epitopes (UIEs) representative of one donor (#7) are shown in black.

Supplementary Figure 1



Supplementary Figure 2