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Original Article

T cell responses to adenoviral vectors expressing the SARS-CoV-2 nucleoprotein

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ABSTRACT

SARS-CoV-2 vaccines aim to protect against COVID-19 through neutralizing antibodies against the viral spike protein. Mutations within the spike's receptor-binding domain may eventually reduce vaccine efficacy, necessitating periodic updates. Vaccine-induced immunity could be broadened by adding T cell-inducing antigens such as SARS-CoV-2's nucleoprotein (N). Here we describe two replication-defective chimpanzee adenovirus (AdC) vectors from different serotypes expressing SARS-CoV-2 N either in its wild-type form or fused into herpes simplex virus glycoprotein D (gD), an inhibitor of an early T cell checkpoint. The vaccines induce potent and sustained $CD8^+$ T cell responses that are broadened upon inclusion of gD. Depending on the vaccine regimen booster immunizations increase magnitude and breadth of T cell responses. Epitopes that are recognized by the vaccine-induced T cells are highly conserved among global SARS-CoV-2 isolates indicating that addition of N to COVID-19 vaccines may lessen the risk of loss of vaccine-induced protection due to variants.

KEYWORDS: vaccine, SARS-CoV-2, nucleoprotein, T cells, epitopes.

INTRODUCTION

The severe acute respiratory syndrome coronavirus (SARS-CoV)-2 crossed into humans towards the end of 2019 and caused a global pandemic with more than 160 million cases and 3.3 million fatalities by May of 2021. The pandemic was initially controlled by lockdowns and government-mandated social distancing and mask wearing [1]. Within a few months vaccines that expressed the SARS-CoV-2's spike protein were developed and entered clinical trials. RNA vaccines from Pfizer [2] and Moderna [3] and adenovirus (Ad) vector vaccines from Johnson & Johnson [4], AstraZeneca [5], and the Gamaleya Institute [6] were shown to be highly efficacious at preventing disease and death and were granted emergency use authorization in different countries. Regions with access to vaccines rapidly set up mass vaccination campaigns, which are reducing the spread of SARS-CoV-2.

It is expected that SARS-CoV-2 will continue to circulate necessitating periodic booster immunizations. The timing of additional doses of vaccine will depend on the longevity of vaccine-induced protective immune responses and their robustness against evolving viral variants. Upon natural infections with coronaviruses, antibody titers decline rapidly [7], rendering individuals potentially susceptible to reinfection. We currently do not know the longevity of antibody responses following immunizations with RNA vaccines [8]. In the same token durability of Ad vector-induced antibody responses in humans remains ill-defined although pre-clinical studies in nonhuman primates have shown them to be sustained for well over a year [9].

T cell responses are prolonged after infections with SARS-CoV-2 infection [10], and memory

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T cells can persist for the lifespan of an individual [11]. SARS-CoV-specific $CD8^+$ T cells, although unable to prevent an infection, can blunt severity of disease, accelerate virus clearance, and reduce spreading [12, 13]. T cells are commonly directed against internal structural or non-structural proteins, which are not subjected to selection pressure by neutralizing antibodies [14] and may thereby prevent loss of vaccine efficacy due to viral mutations.

Here we describe two replication-defective AdC vectors based on serotypes SAd-V23 (referred to as AdC6) and SAd-V24 (referred to as AdC7) expressing N of an early SARS-CoV-2 isolate either in its wild-type form or fused into herpes simplex virus (HSV-1) glycoprotein D (gD), which blocks an early T cell checkpoint. HSV-1 gD binds to the herpes virus entry mediator (HVEM) expressed on antigen-presenting cells and thereby prevents its binding to the B and T lymphocyte attenuator (BTLA), which is carried by cells of the adaptive immune system and upon ligation dampens signaling downstream of the T and B cell receptors [15]. Blockade of BTLA-HVEM interactions in turn leads to enhanced and broadened T cell responses to not only immunodominant but also subdominant epitopes [16, 17].

The AdC vaccines were generated, and quality controlled and then tested in mice for induction of T cell responses using prime or prime-boost regimens. As expected, inclusion of gD enhanced the breadth of $CD8^+$ T cell responses. Depending on the vaccine regimen responses further increased upon booster immunizations. The sequences of epitopes that were recognized by vaccine-induced T cells were conserved among different SARS-CoV-2 isolates from around the globe supporting our notion that inclusion of an N component into COVID-19 vaccines might guard against loss of protection due to viral variants.

MATERIALS AND METHODS

Cell lines

HEK-293 cells and CAR-transduced CHO cells were maintained in Dulbecco's Modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. RMA-S cells were grown in DMEM supplemented with 2% FBS and 0.05 M of 2-mercaptoethanol.

Construction and quality control of the AdC vectors

The cDNA sequence for N of SARS-CoV-2 within the paT7-N plasmid was kindly provided by the laboratory of Dr. Elledge, Massachusetts General Hospital, Boston, MA (Supplementary Table 1). The paT7-N plasmid was digested with ApaI and NotI, and the N sequence was cloned into the backbone plasmid pShCMV-eGFP, which had been digested by ScaI and NotI, thus replacing eGFP and resulting in pShCMV-N. $N_{1\text{-}233}$ which expresses the N-terminal 233 amino acids was generated from pShCMV-N upon digestion with PvuII and ScaI followed by self-ligation; $N_{235\text{-}420}$, which expresses the 214 amino acid long C-terminal part of the protein was formed by cutting pShCMV-N with AgeI and PvuII.

To generate the gDN fusion gene, the pShCMV-gD was used as the backbone plasmid. PCR cloning strategy was used to remove start and stop codons of the N gene with the forward primer: 5'-GCGG GCCCTCCGATAACGGCCCACAAAATC-3' and the reverse primer: 5'-GCGGGCCCGGCCTGAG TACTATCTGCAG-3'; pShCMV-gD and the N gene PCR product were digested by ApaI enzyme and then ligated resulting in pSh-gDN plasmid.

The expression cassettes, which carry the N or gDN genes under the control of the cytomegalovirus (CMV) immediate early (IE) enhancer and the CMV promoter followed by an intron to improve expression and terminated by the bovine growth hormone polyadenylation (BGH polyA) signal, were excised from the pSh-CMV plasmids and cloned into the viral molecular clones of AdC6 and AdC7 using the rare restriction enzyme sites for I-CeuI and PI-SceI. Each cloning step was verified by restriction enzyme digest and sequencing of the insertion sites. The recombinant viral molecular clones were linearized and transfected into HEK-293 cells. Once viral plaques developed, cells were harvested, virus was released and expanded over several rounds on HEK-293 cells. Upon purification by CsCl gradient centrifugation AdC vectors were formulated in 2.5% Glycerol/25mM NaCl/20mM TRIS buffer, pH 8.0. Virus particle content was determined by spectrophotometry and infectious units were measured by a nested reverse transcription (RT)-polymerase chain reaction (PCR) conducted with RNA of HEK-293 cells that had been infected for 7 days with serial dilutions of

the vectors. Results showed that both yields and virus particle to infectious unit ratios were within acceptable ranges. Genetic integrity of the AdC vector genome was determined by restriction enzyme digest of purified viral DNA followed by gel electrophoresis. Genetic stability was established using the same procedure with viruses that had been passaged 12 times sequentially on HEK-293 cells. The AdC vectors passed the quality control assays.

Protein expression

The AdC vectors were tested for protein expression upon transfection of HEK-293 cells or CHO cells stably transfected to express CAR. Briefly, 1×10^6 cells/flask were infected for 48 hours with ~1000 vp/cell of the AdC6 or AdC7 vectors expressing N or gDN. Negative control cells were transfected with an AdC vector expressing an unrelated protein. Cells were collected and lysed in Radioimmuno-precipitation assay (RIPA) buffer supplemented with a 1% µl protease inhibitor (Santa Cruz Biotechnology Inc., Dallas, TX). The lysate was stored at −80 °C until further use. 15 µl of protein sample was resolved on 12% SDS-PAGE and transferred to a polyviny-lidene difluoride (PVDF) membrane (Merck Millipore, Burlington, MA). The membrane was blocked in 5% powder milk (blocking buffer) overnight at 4 °C. The primary antibody to gD (clone PA1-30233, Invitrogen, Carlsbad, CA) diluted to 1:1000 in blocking buffer was added for 1 hr at room temperature. Anti-SARS-CoV-2 N (rabbit) antibody (Rockland; Cat# 200-401-MS4) was used at dilution of 1:1000 followed by HRP-conjugated goat anti-rabbit IgG (Abcam; Cat# ab6721) at a dilution of 1:10000 for 1 hr. β-Actin protein was detected by β-actin mouse monoclonal IgG antibody (Santa Cruz Biotechnology; Cat# Sc-47778) at dilution of 1:1000, followed by HRP-conjugated Goat anti-mouse IgG (Sigma; Cat# SAB3701047) at dilution of 1:5000 for 1 hr.

Membranes were washed with 1X TBS-T prior to incubating with HRP-conjugated goat anti-rabbit secondary IgG (ab6721, Abcam, Cambridge UK) for 1 hr at room temperature. Membranes were washed 3 times with 1X TBS-T. The developing agent Super Signal West Pico Chemiluminescent (Thermo Fisher Scientific, Waltham, MA) was added. Membranes were shaken in the dark for 5 min, dried and developed.

Liquid chromatography tandem mass spectrometry

AdC-gDN-infected cells (1000 vp/cell for 24 hr) were lysed, and sample proteins were run through a 12% SDS-PAGE gel. Gel regions (55 to 100 kDa) were excised, reduced with tris(2-carboxyethyl) phosphine (TCEP), alkylated with iodoacetamide, and digested in-gel with trypsin. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis was performed by the Proteomics and Metabolomics Facility at The Wistar Institute using a Q Exactive HF mass spectrometer (ThermoFisher Scientific) coupled with an UltiMate 3000 nano UPLC system (Thermo Scientific). Samples were injected onto a PepMap100 trap column (0.3×5 mm packed with 5 µm C18 resin; Thermo Scientific), and tryptic peptides were separated by reversed phase high-performance liquid chromatography (HPLC) on a BEH C18 nanocapillary analytical column (75 µm i.d. x 25 cm, 1.7 µm particle size; Waters) using a 2 hr gradient formed by solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). Eluted peptides were analyzed by the mass spectrometer set to repetitively scan m/z from 400 to 1500 in positive ion mode. The full MS scan was collected at 60,000 resolution followed by data-dependent MS/MS scans at 15,000 resolution on the 20 most abundant ions exceeding a minimum threshold of 20,000. Peptide match was set as preferred; options enabled were to exclude isotopes and charge-state screening to reject singly and unassigned charged ions.

Peptide sequences were identified using MaxQuant 1.6.15.0 [18]. MS/MS spectra were searched against a UniProt human protein database (10/10/2019) using full tryptic specificity with up to two missed cleavages, static carboxamidomethylation of Cys, variable oxidation of Met, deamidation of Asn, and protein N-terminal acetylation. Consensus identification lists were generated with false discovery rates of 1% at protein, and peptide levels.

Mice

Female 6-week-old C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed at the Animal Facility of the Wistar Institute and treated according to approved protocols. Unless stated otherwise experiments were conducted with groups of 5 mice 2 or 3 times.

Vaccination and infection of mice

AdC6 or AdC7 vectors were diluted in sterile saline. A total volume of 200 µl containing the indicated numbers of vp was injected intramuscularly into the left hindleg of mice.

Preparation of splenocytes

Spleens were harvested from mice. Single cell suspension was generated by mincing spleens with mesh screens in Leibovitz's L15 medium followed by passing cells through a 70 µm filter (Thermo Fisher Scientific). Red blood cells were lysed by 1 x RBC lysis buffer (eBioscience, San Diego, CA).

In vitro stimulation of lymphocytes

Lymphocytes were stimulated with pools of peptides or individual peptides (GenScript USA Inc; purity 90%). Peptides were 15 amino acids in length and overlapped by 10 amino acids with the adjacent peptides. Individual peptides were diluted according to the manufacturer's instructions in either water, DMSO, ammonia water, formic acid or N-methyl. For stimulation ~10^6 lymphocytes plated in medium containing 2% FBS and Golgiplug (BD Bioscience; San Jose, CA), at 1.5 µl/ml were cultured with the peptide pools or individual peptides, each present at a final concentration of 2 µg/ml, for 5 hr at 37 °C in a 5% CO_2 incubator. Control cells were cultured without peptides.

Intracellular cytokine staining (ICS) and analyses by flow cytometry

Following stimulation cells were incubated with anti-CD8-APC (clone 53-6.7, BioLegend, San Diego CA), anti-CD4-PerCp5 (clone Gk1.5, BioLegend), anti-CD44-Alexa Flour 700 (clone IM7, BioLegend) and violet live/dead dye (Thermo Fisher Scientific) at 4 °C for 30 min in the dark. Cells were washed once with PBS and then fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences, San Jose, CA) for 20 min. Following fixation, cells were incubated with an anti-INF-γ-FITC antibody (Clone, XMG1.2 BioLegend) at 4 °C for 30 min in the dark. Cells were washed and fixed in 1:3 dilution of BD Cytofix fixation buffer (BD Pharmingen, San Diego CA). They were analyzed by a BD FACS Celesta (BD Biosciences, San Jose, CA) and DiVa software. Post-acquisition analyses were performed with FlowJo (TreeStar, Ashland, OR). Data shown in graph represents % $CD8^+$ or $CD44^+CD8^+$ cells producing INF-γ upon peptide stimulation. Background values obtained for the same cells cultured without peptide(s) were subtracted.

RMA-S assay

$5x10^5$/well RMA-S cells were seeded in DMEM supplemented with 2% FBS and 0.05 M of 2-mercaptoethanol and incubated overnight at 37 °C. 100 µl of individual N peptides were added at $5x10^{-5}$ M into each well. Additional cells were incubated with the peptide pools (positive control) and no peptides (negative control). Cells were incubated with peptides for 6 hr at 37 °C. Then media was discarded and replaced with an H-$2K^b$ antibody conjugated to PE (BD Pharmingen; Cat# 553570) and an H-$2D^b$ antibody conjugated to FITC (BD Pharmingen; Cat# 553573) at a dilution of 1:100 in cell staining buffer (Biolegend; Cat# 420201). Live/Dead fixable violet dead cell stain (Invitrogen by Thermo Fisher Scientific; Lot# 2256722) was used at a dilution of 1:400 to determine cell viability; 50 µl/well of this staining dilution was added, and cells were incubated for 1 hr at 4 °C. Cells were washed twice with 150 µl of cell staining buffer (2x), followed by resuspension in 1x stabilizing fixative (BD Stabilizing Fixative; REF# 338036). The level of H-$2K^b$ and H2-D^b binding to each individual peptide was detected by flow cytometry.

Analysis of conservation of sequences between different unique SARS-CoV-2 isolates

For each geographic region data were acquired from the database of the National Center for Biotechnology Information using the keywords 'Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)', 'taxid:2697049' for virus and 'nucleocapsid phosphoprotein' for protein. The analyses included data available by May 18, 2021. Using Excel, duplicates and incomplete sequences were removed, and the remaining sequences were screened for the presence of 15 mer peptide sequences.

RESULTS

AdC vectors

We constructed six AdC vectors expressing SARS-CoV-2 N. They included AdC6 and AdC7 expressing the full-length protein in its wild-type

form (AdC6-N, AdC7-N) or fused into HSV-1 gD (AdC6-gDN, AdC7-gDN). Three truncated versions of N were expressed by AdC6: $N_{1\text{-}233}$ which expresses the N-terminal 233 amino acids (AA) and $N_{235\text{-}420}$ which expresses the 214 AA long C-terminal part of the protein and $N_{1\text{-}137,229\text{-}420}$, which lacks 92 AA in the central part of N.

Protein expression by vectors expressing the full-length wild-type N was confirmed by Western Blot analysis using an N-specific antibody or, for vectors carrying N within gD, an antibody to gD (Fig. 1a, b). Results with the latter antibody were confirmed by liquid chromatography with tandem mass spectrometry, which revealed the presence of multiple N-derived polypeptides spanning the entire sequence of the protein (Fig. 1c, d).

Initially, to confirm immunogenicity of N as presented by the AdC vectors, groups of 5 C57Bl/6 mice were immunized with 2×10^{10}vp of AdC6-N or AdC6-gDN. Naïve mice served as controls.

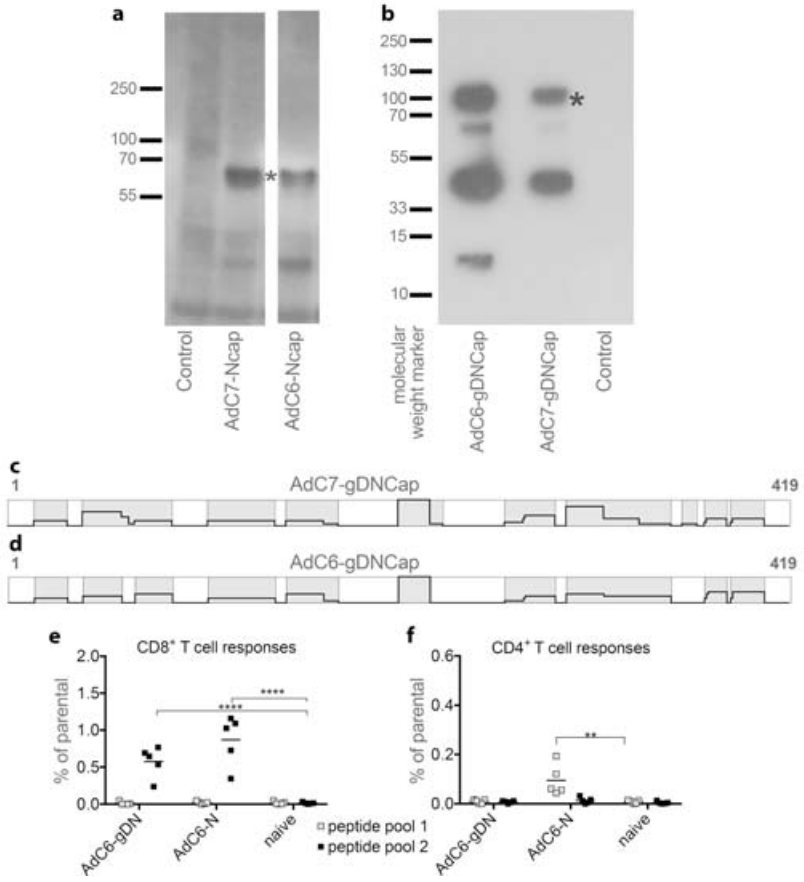

Fig. 1. Expression of the transgene products and T cell responses to N. [a, b] Western blots for AdC7-N and AdC6-N probed with an antibody to N [A] and AdC7-gDN and AdC6-gDN [b] probed with an antibody to gD. Ad vectors expressing an unrelated protein were used as controls. * indicates the N protein in its wild-type form or fused into gD. Protein loading was controlled for by a subsequent stain with an antibody to ß-actin (not shown). [c, d] Liquid chromatography with tandem mass spectrometry. The diagrams in C and D are a graphical representation of the protein expressed by AdC7-gDN [c] and AdC6-gDN [d] from amino acid residue 1 to 419. Protein sequences identified by LC-MS/MS are indicated by the grey boxes. The black trace shows the MS/MS spectra count of the identified peptides relative to the peptide with the highest spectra count. $CD8^+$ [e] and $CD4^+$ [f] T cell responses to the two peptide pools are shown as % IFN-γ^+ cells/over all cells of the subset. Statistical differences calculated by multiple t-tests are indicated with lines with stars above. (****) – p value < 0.0001; (**) – p-value between 0.001-0.01.

Splenocytes were tested for responses to two pools, which each contained 41 peptides spanning the sequence of N. Peptides were 15 AA in length and overlapped by 10 AA with the adjacent peptide. Pool 1 contained the peptides of the C-terminal half of N, and pool 2 contained those of the N-terminal part. Splenocytes were tested for responses upon a brief *in vitro* stimulation with the peptides followed by staining for T cell markers and intracellular interferon (IFN)-γ. Both vaccines induced robust $CD8^+$ T cell responses to pool 2 while $CD4^+$ T cells were only detectable in AdC6-N immunized mice; they were directed to peptides within pool 1 (Fig. 1e, f).

Epitope specificity of T cells induced by AdC-N or AdC-gDN vector priming

To determine the breadth of N-specific T cell responses, pooled splenocytes from vaccinated mice were stimulated with individual peptides spanning the N sequence and then stained for CD4, CD8 and CD44 surface markers and intracellular IFN-γ. Splenocytes of naïve mice served as controls and cells cultured with medium rather than peptides were used to determine background activity. Frequencies of $CD44^+CD8^+$ or $CD44^+CD4^+$ T cells producing IFN-γ in the absence of peptides were subtracted from frequencies of the same cell subsets that produced IFN-γ in response to an N peptide.

Splenocytes from naïve mice had marginal responses and only the frequency of $CD44^+CD4^+$ T cells against peptides 64 slightly exceeded 0.05% (Supplemental Fig. 1a). We therefore set the limit of positive responses at frequencies of or above 0.2%. Accordingly, the Y-axis of each of the following graphs starts at 0.2. To further simplify the graphs results are only shown for peptides that scored positive for a given T cell subsets in at least one set of experiments. As additional controls we tested mice that were immunized with Ad vectors which expressed only the N or C terminal part of N; lymphocytes were tested with peptides to the deleted parts. Vectors failed to elicit a response to sequences that were not present in the vaccine insert (Supplemental Fig. 1b). To control for gD mice that had been injected 4 weeks earlier with an AdC6 vector expression gD fused with an irrelevant antigen were tested and again shown to be negative (Supplemental Fig. 1c).

To determine $CD8^+$ T cell epitopes that were recognized after priming, groups of 5 C57Bl/6 mice were immunized with 2×10^{10}vp of AdC6-N, AdC7-N, AdC6-gDN or AdC7-gDN and tested 2 weeks later. Another group was immunized with 1×10^{10}vp of AdC6-gDN and tested 3 months later to determine response durability. Responses to AdC6-N and AdC7-N were monospecific and solely directed against peptide 44 (Fig. 2a, d). Inclusion of gD into the vaccines broadened T cell responses to additional peptides although the response to peptide 44 remained dominant (Fig. 2b, e). Reducing the AdC6-gDN dose to 1×10^{10} and delaying testing of splenocytes to 3 months after vaccination caused a further increase in the magnitude and breadth of the response as well as a shift in immunodominance away from peptide 44 towards other epitopes that were not recognized when T cells were tested at 2 weeks after vaccination (Fig. 2c, f).

Inclusion of gD did not increase the overall magnitude of $CD8^+$ T cell responses when they were tested 2 weeks after immunization as shown by sum of responses to 'unique' epitopes (Fig. 2d-f). Sum of responses to all peptides excluded those to adjacent peptides that express the same epitope according to epitope predication (http://tools.iedb.org/main/). Frequencies were highest when tested 3 months after vaccination. This unusual kinetics was only seen for vaccines expressing the gDN insert. When mice vaccinated with AdC6N at a moderate dose of 2×10^{10}vp were tested 10 weeks after immunization, the response remained monospecific for peptide 44 and frequencies were slightly lower than when mice were tested after 2 weeks (5.6% vs. 8.9%, data not shown).

Splenocytes from mice injected with AdC6 vectors were tested for $CD44^+CD4^+$ T cell responses. $CD4^+$ T cell responses to AdC6-N (Fig. 3a, d) and AdC6-gDN (Fig. 3b, e) were low and directed to 5 vs. 2 peptides, respectively. Delaying testing till 3 months after AdC6-gDN vaccination augmented frequencies and breadth of $CD4^+$ T cell responses (Fig. 3c, f). Again, this was not seen after immunization with AdC6-N (data not shown).

Epitope specificity of $CD8^+$ T cells induced by AdC-N prime-boosting

Groups of 5 C57Bl/6 mice were primed with AdC6-N at 5×10^{10} or 2×10^{10}vp. They were boosted

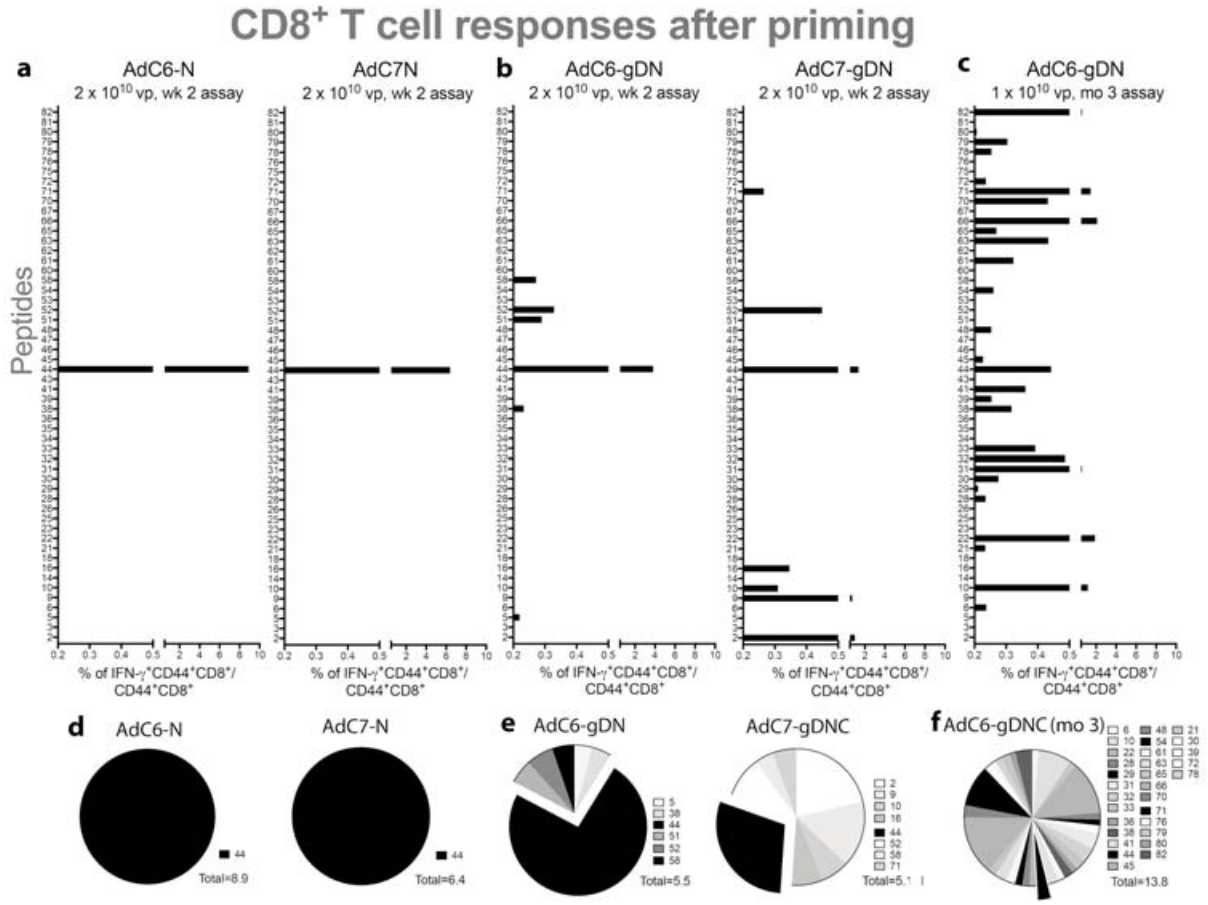

Fig. 2. CD8$^+$ T cell responses after priming. Frequencies of CD8$^+$ T cell responses to N peptides that carry potential epitopes are shown as %IFN-γ $^+$CD44$^+$CD8$^+$ cells/CD44$^+$CD8$^+$ cells. Background responses were subtracted. The upper graphs show frequencies, the lower graphs show the relative distribution of responses to individual peptides. The numbers below the pie charts show the sum of frequencies (excluding those of positive adjacent peptides that according to epitope prediction express the same epitope) as indicators for response magnitude; legends indicate the peptide number to which responses were elicited. Those close to the pie charts were used to derive the sum of the response, those to the right were excluded. [a, d] Responses to the AdC-Nap vector; [b, e] responses to the AdC-gDN vector. For both sets of data mice were immunized with 2×10^{10}vp of vector and splenocytes were analysed 2 weeks later. [c, f] show responses to the AdC6-gDN vector given at 1×10^{10}vp/mouse. Splenocytes were analysed 3 months later.

6 weeks or 2 months later with the same doses of AdC7 expressing the same insert. An additional group was immunized first with 2×10^{10}vp of AdC7-N and then boosted 2 months later with the same dose of AdC6-N. Splenocytes were tested 2 weeks after the boost for production of IFN-γ to N peptides as described above. With high doses of AdC6-N and AdC7-N, peptide 44 and the adjacent peptide 45 remained immunodominant and only one additional peptide (i.e., peptide 21) scored a low response after the boost given within a 6-week interval (Fig. 4a, c). Reducing the vaccine dose to 2×10^{10}vp and extending the interval between the two injections to 2 months increased magnitude and breadth of N-specific CD8$^+$ T cell responses above those seen after priming (Fig. 4b, d).

Epitope specificity of CD8$^+$ T cells induced by AdC-gDN prime-boosting

A similar experiment was conducted with AdC-gDN. One group was primed with 5×10^{10}vp of AdC7-gDN and boosted 6 weeks later with the same dose of AdC6-gDN. Splenocytes were tested 2 weeks later (Fig. 5a, e). The next group was primed with the same dose of AdC6-gDN; boosting with AdC7-gDN was delayed till week 8 and splenocytes

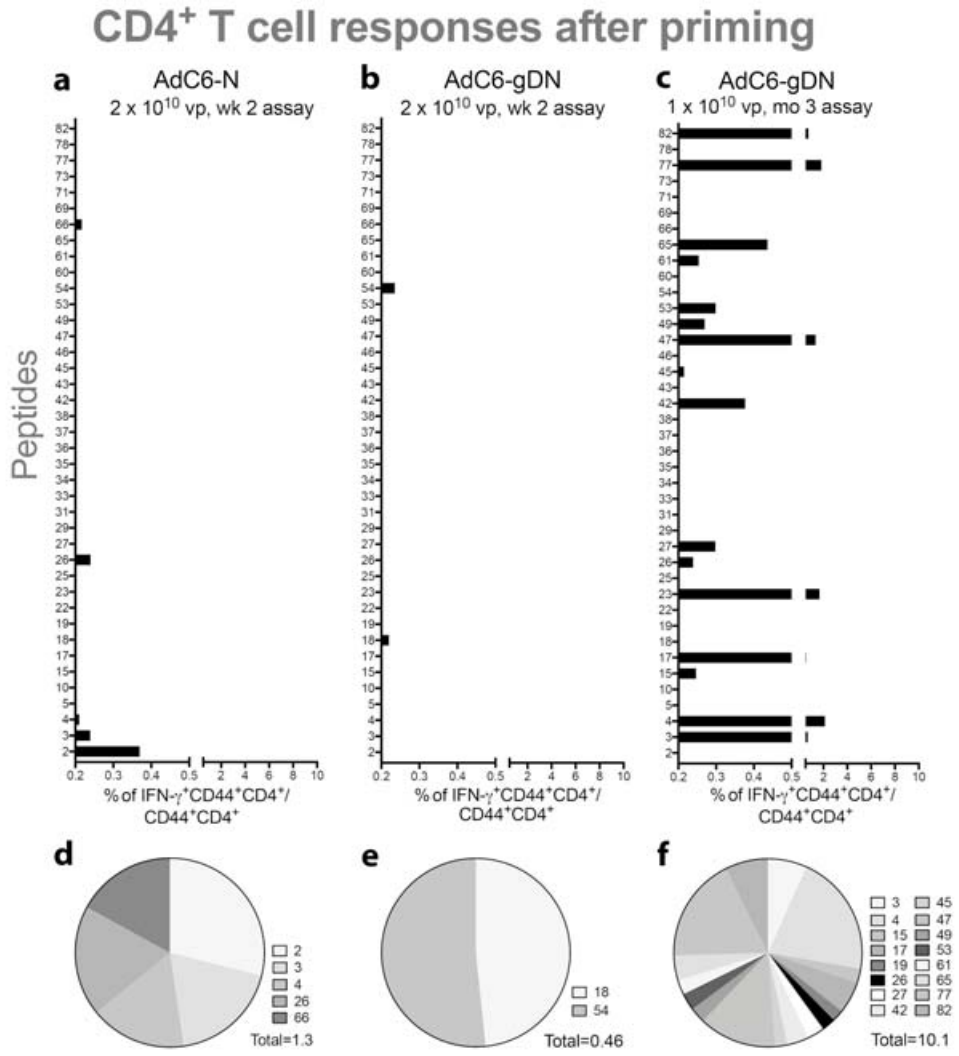

Fig. 3. CD4$^+$ T cell responses after priming. The graphs show frequencies and relative distribution of responses to individual peptides as in Fig. 2 for mice immunized with 2×10^{10}vp of the AdC6-N [a, d] or AdC6-gDN [b, e] vectors analysed 2 weeks later or to 1×10^{10}vp of the AdC6-gDN vector [c, f] analysed 3 months later.

were tested 3 months after the boost (Fig. 5b, f). Both regimens induced responses that were dominated by those to peptides 44 and 45. The latter group also showed robust responses to three additional peptides and had overall higher frequencies of IFN-γ-producing CD8$^+$ T cells. One additional group was primed with 5×10^9vp of the AdC7-gDN and boosted 4 weeks later with the same dose of the AdC6-gDN vector; splenocytes were tested 6 weeks later (Fig. 6c, g). A fourth group was primed with 2×10^9vp of AdC6-gDN, boosted 4 weeks later with AdC7-gDN and tested 4 months later (Fig. 6d, h). The breadth and magnitude of the CD8$^+$ T response was markedly higher at the lower vector doses although they failed to exceed those of responses after a single immunization (Fig. 2).

Epitope specificity of CD4$^+$ T cells induced by AdC-gDN prime-boosting

N-specific CD4$^+$ T cells were analyzed upon prime-boosting with AdC-gDN vectors. The 5×10^{10}vp dose of AdC vectors with a 6-week interval between prime and boost resulted in low and narrow responses by 2 weeks after the boost (Fig. 6a, e). Reversing the order of the vectors, increasing the interval between the two injections to 8 weeks and testing 3 months after the final

Table 1a. AA sequences for peptides of SARS-Co-V2 N.

Peptide	Amino Acids	MHC class I	Rank	% Conserved sequences					
				Africa	Asia	Europe	North America	Oceania	South America
2	6–20	PQNQRNAPRITFGGP	1.1	84.14	87.35	90.99	86.38	93.45	91.67
9	41-55	RPQGLPNNTASWFTA	0.9	100.00	99.21	100.00	97.82	97.02	100.00
10	46-60	PNNTASWFTALTQHG	0.08	100.00	99.21	100.00	98.39	95.83	100.00
21	101-115	MKDLSPRWYFYYLGT	0.82	99.31	99.21	99.10	98.78	99.40	100.00
30	146-160	IGTRNPANNAAIVLQ	0.11	100.00	96.05	93.69	96.24	95.83	100.00
31	151-165	PANNAAIVLQLPQGT	1.4	97.93	94.86	93.69	95.35	95.83	100.00
32	156-170	AIVLQLPQGTTLPKG	0.27	97.93	96.44	99.10	96.50	97.02	94.44
44	216-230	DAALALLLLDRLNQL	0.19	94.48	96.44	92.79	96.97	95.83	100.00
45	221-236	LLLLDRLNQLESKMS	0.78	85.52	86.17	77.48	67.72	95.24	91.67
61	301-315	WPQIAQFAPSASAFF	0.18	100.00	98.81	100.00	98.35	98.81	94.44
63	311-326	ASAFFGMSRIGMEVT	0.23	96.55	98.02	100.00	97.44	97.02	94.44
66	326-340	PSGTWLTYTGAIKLD	0.32	97.93	95.65	93.69	95.05	95.24	97.22
67	331-345	LTYTGAIKLDDKDPN	0.32	97.93	97.23	96.40	96.45	97.02	97.22
70	346-360	FKDQVILLNKHIDAY	1.5	97.93	97.63	100.00	97.51	98.21	100.00
71	351-365	ILLNKHIDAYKTFPP	0.54	96.55	98.02	95.50	94.63	97.02	94.44
81	401-415	DDFSKQLQQSMSSAD	0.71	96.55	94.86	95.50	94.35	97.02	100.00
Peptide	**Amino Acids**	**MHC class I**	**Adj. Rank**	**Africa**	**Asia**	**Europe**	**N. America**	**Oceania**	**S. America**
3	11–25	NAPRITFGGPSDSTG	15.08	100.00	98.81	100.00	98.35	98.81	94.44
22	106-120	PRWYFYYLGTGPEAG	0.94	100.00	98.42	97.30	97.97	98.21	94.44
23	111-125	YYLGTGPEAGLPYGA	6	100.00	98.42	97.30	97.42	97.02	94.44
61	301-315	WPQIAQFAPSASAFF	0.31	97.93	95.65	93.69	95.05	95.24	97.22
66	326-340	PSGTWLTYTGAIKLD	8.56	86.21	86.96	90.99	87.90	93.45	88.89
82	406-420	QLQQSMSSADSTQA	19.38	97.93	96.05	97.30	93.62	97.62	100.00
Number of total sequences**				1572	3032	1127	260737	13299	590
Number of unique sequences***				305	465	144	15017	899	46
Number of complete unique sequences****				145	253	111	6710	168	36

*High mutation rates are highlighted
**NCap sequences available through the database of the National Center for Biotechnology Information on May 18, 2021
***Unique sequences within the dataset
****Complete sequences within the set of unique sequences

Table 1b. AA sequence 221-235 of SARS-CoV-2 N.

Peptide 45 Sequence 221-235**	Frequencies of mutations within peptide 45 (SARS-Co-V2 N 221-235)*					
	Africa	Asia	Europe	North America	Oceania	South America
LLLLDRLNQLESKMS	85.52	86.17	77.48	67.72	95.24	91.67
LLLLDRLNQLESKM<u>F</u>	9.66	6.72	15.32	15.47	0.00	2.78
LLLLDRLNQLESK<u>I</u>S	2.76	4.74	6.31	14.29	0.60	5.56
LLLLDRLNQ<u>F</u>ESKMS	0.00	0.00	0.90	0.21	1.79	0.00
LLLLDRLN<u>H</u>LESKMS	0.00	0.79	0.00	0.36	0.60	0.00

*Table shows the wildtype AA sequence 221-235 of SARS-CoV-2 N in row 3 followed by the most frequent variants and their frequencies in different regions.
**Mutated amino acids are underlined

Table 1c. AA types and frequencies of mutations.

Amino acid position	Wt*	Types and frequencies of mutations		
		Mutations**	Number of isolates***	% of isolates****
			5165	**69.62**
221	L	F, V	14	0.19
222	L	M	2	0.03
223	L			
224	L	F, P	5	0.07
225	D	Y, H, E	3	0.04
226	R	K	8	0.11
227	L	F, V	11	0.15
228	N	H, S, R, L	9	0.12
229	Q	F, H, L, K	18	0.24
230	L	F	25	0.34
231	E	T, D	2	0.03
232	S	G, I, N, R, C	62	0.84
233	K	I, R	12	0.16
234	M	I, V, L, T	1019	13.74
235	S	F, L, P, A, C, Y	1130	15.23

*wt – wild-type, the column shows the AA sequence of SARS-CoV-2 N in the original isolate
**The column shows the AA changes at the different positions in all sequences from Table 1A
***The column shows number of sequences with mutations at the different positions, numbers of unmodified sequences are shown on top
****The column provides the same information as the column to the left in percent of isolates over all unique complete sequences.

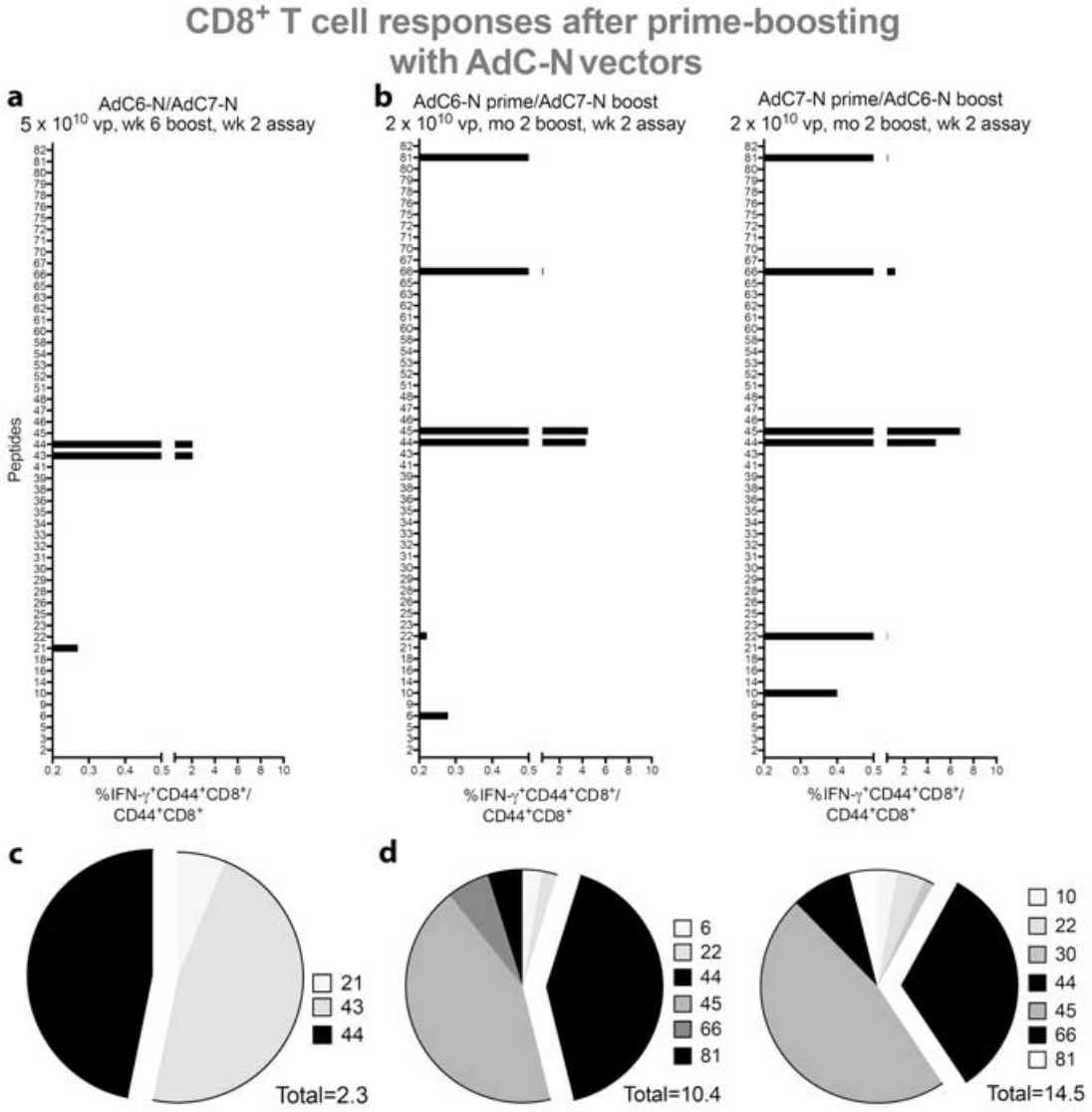

Fig. 4. CD8$^+$ T cell responses to AdC-N prime-boost regimens. The graphs show frequencies and relative distributions of CD8$^+$ T cells to individual peptides as in Fig. 2 using splenocytes from mice that had been primed with AdC6-N at 5 or 2×10^{10}vp and were boosted 6 weeks [a, d] or 2 months [b, e] later with the corresponding AdC7 vector. An additional group was primed with 1×10^{10}vp of AdC7-N and boosted 2 months later with AdC6-N. All assays were conducted 2 weeks after the boost.

injection diminished the response further (Fig. 6b, f). Reducing the vector dose to 5×10^9vp per injection and boosting after 4 weeks induced strong and broad CD4$^+$ T cell responses when tested 6 weeks after the last injection (Fig. 6c, vg). Similar results were obtained with a vaccine regimen where the order of the vectors used at the same doses was reversed and splenocytes were tested 4 months after the 2nd injection (Fig. 6d, h).

MHC class I epitope binding and MHC class I and II epitope prediction

We tested each peptide for binding to RMA-S cells [19], which lack the transporter associated with antigen processing and therefore fail to express MHC class I antigens unless a D^b or K^b-binding peptide is added to stabilize surface expression of MHC class I molecules, which can then be detected by staining and flow cytometry.

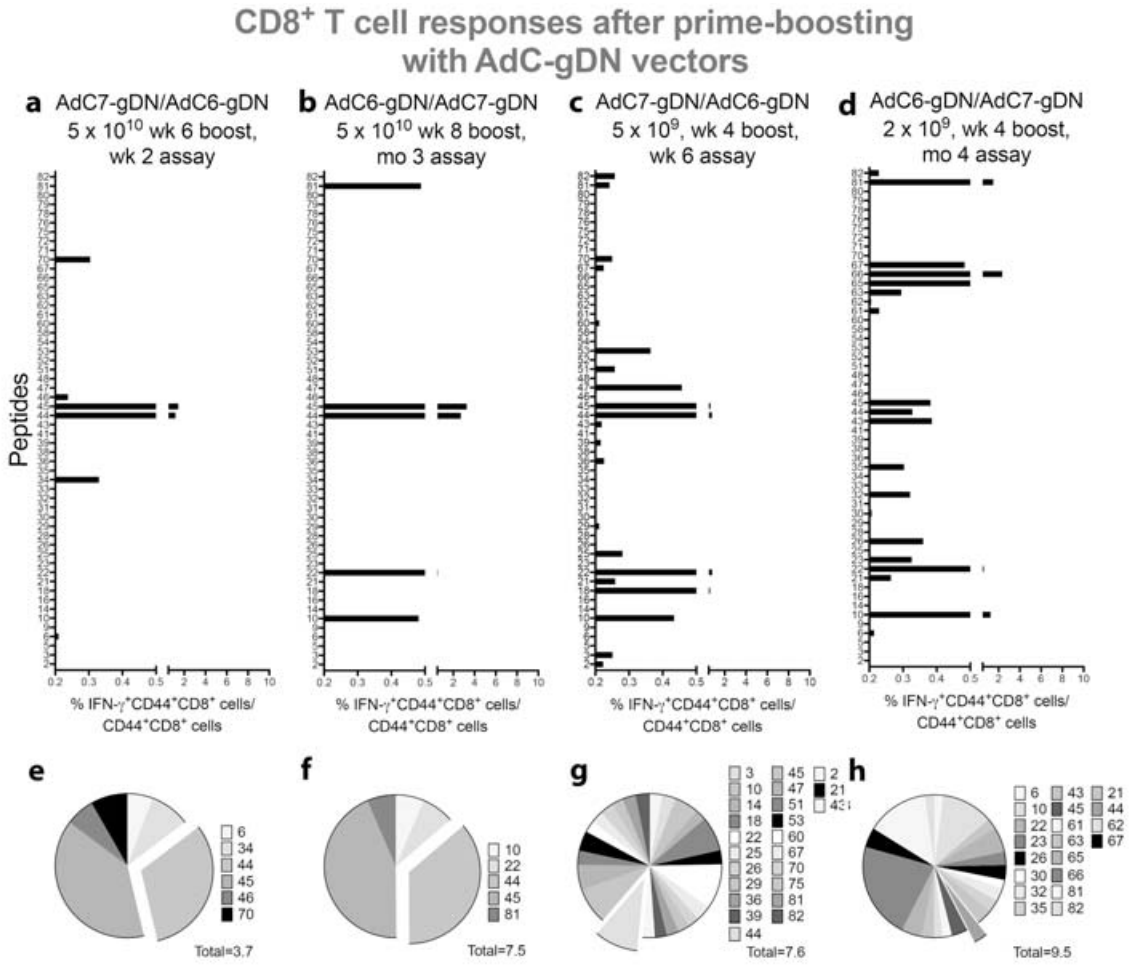

Fig. 5. CD8$^+$ T cell responses to AdC-gDN prime-boost regimens. The graphs show frequencies and relative distribution of CD8$^+$ T cells to individual peptides as in Fig. 2 using splenocytes from mice that had been primed with AdC7-gDN [a, c, e, g] or AdC6-gDN [b, f, d, h] at the indicated doses. They were boosted 4 to 8 weeks later as shown in the graph titles with the same dose of the corresponding heterologous vector. Splenocytes were tested 2 [a, e] or 6 [c, g] weeks or 3 [b, f] or 4 [d, h] months later.

In addition, we used epitope predication software (http://tools.iedb.org/main/) to determine which peptides were likely to bind to H-2^b class I or II. Prediction of MHC class I-binding peptides correlated with results obtained with RMA-S cells (see Supplemental Fig. 2) and with most peptides that scored positive in the CD8$^+$ T cell assays. Magnitude of T cell responses was not necessarily linked to high prediction scores or the most pronounced increase in MHC class I expression on RMA-S cells. MHC class II epitope prediction was less foretelling for positive results in the CD4$^+$ T cell assays. We used the same software to assess if results obtained in pre-clinical may have relevance for human T cells. As shown in Supplementary Table 2 most of the peptides recognized by H-2^b mice also scored as HLA binders.

T cell epitope conservation

Viral escape can dampen vaccine efficacy. We determined the degree of changes by analysing 7423 unique and complete isolates from across the globe for the presence of the epitopic N peptide sequences. The AA sequences for peptides that identified the different epitopes were highly conserved (Table 1a). North America, the source of most sequences, showed the highest degree of variability for both CD4$^+$ and CD8$^+$ T cell epitopes while isolates from South America and Oceania were the least variable. One peptide,

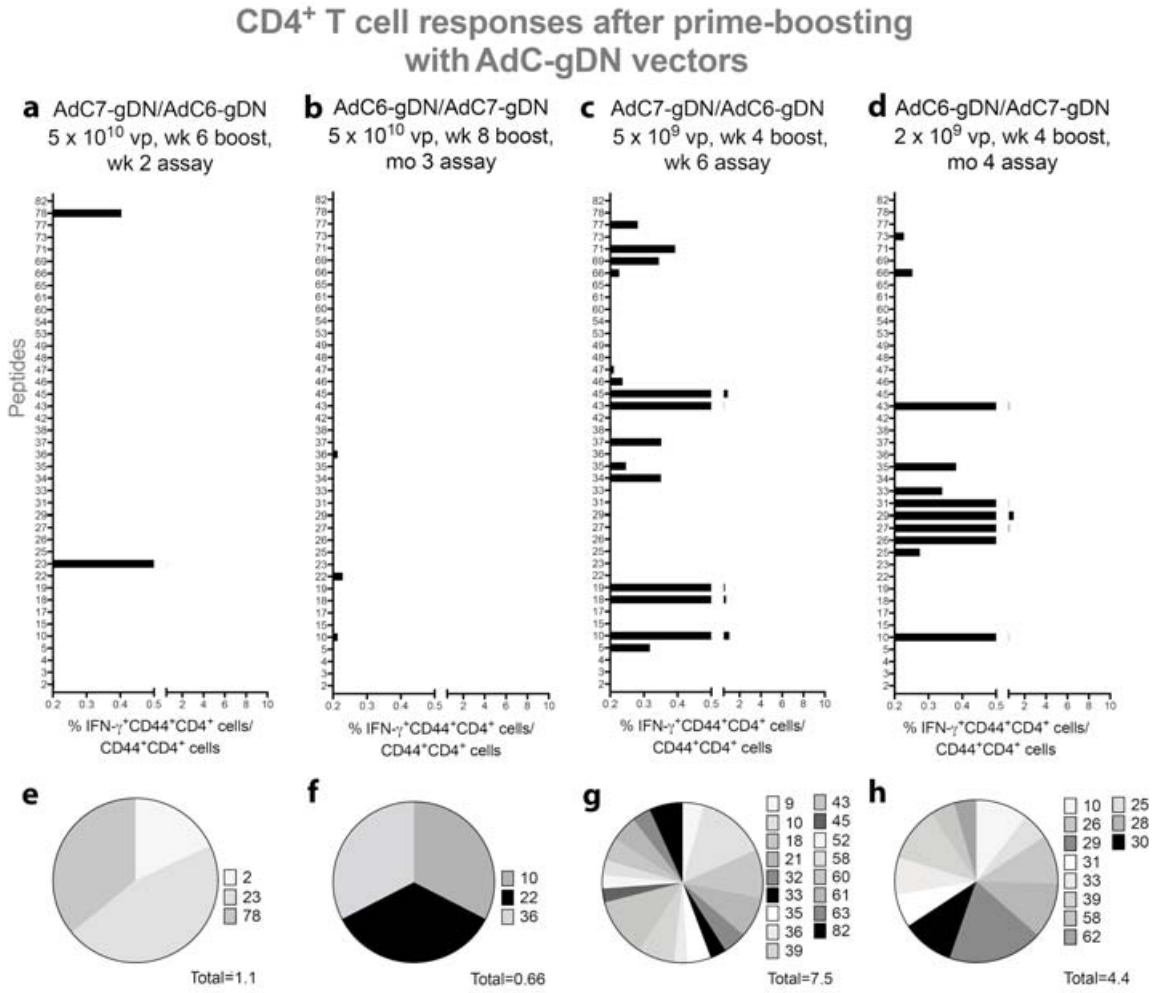

Fig. 6. CD4$^+$ T cell responses to AdC-gDN prime-boost regimens. The graphs show frequencies and relative distribution of CD4$^+$ T cells to individual peptides as in Fig. 2 using splenocytes from mice that had been primed, boosted, and tested as described in legend to Fig. 5.

peptide 45, which represents AA 221-235 of N, showed high variability in >30% of North American and >20% of European SARS-CoV-2 isolates. We further analysed the variability of the 221-235 sequence within the available sequence set. In most regions, but for North America and Europe, variability of individual amino acids within sequence 221-235 mirrored the overall variability of N. The most common mutations in all regions but for Oceania and South America were a serine to phenylalanine exchange in position 235 or a methionine to isoleucine exchange in position 234 (Table 1b). We screened the unique sequences for the position of changes within the N 221-235 sequence. Over 90% of the mutations involved AA 234 and 235 (Table 1c). An analyses with epitope prediction software showed that sequence 221-235 is expected to bind to HLA-A*02:01, HLA-A*02:03, HLA-A*02:08 and HLA-B*08:01 with a rank of 0.8-0.9; nevertheless, the core epitope (LLLDRLNQL) for these HLA types does not involve the commonly mutated AA 234 and 235, which are also unlikely to play a role in binding to human MHC class II molecules (http://tools.iedb.org/main/).

DISCUSSION

Vaccines can stop a pandemic. Those based on attenuated viruses tend to be highly effective. They induce a full spectrum of adaptive immune responses unlike inactivated viruses or protein

vaccines that in general stimulate antibodies and $CD4^+$ T cells but are poor inducers of $CD8^+$ T cells. Most of the inactivated viral vaccines require periodic booster immunizations because protective immunity wanes [20], or the virus escapes by accumulating mutations [21]. Genetic vaccines including RNA vaccines or E1-deleted Ad vectors are perceived by the adaptive immune system like attenuated viruses; they induce de novo synthesis of the vaccine antigen, which promotes its processing for association of immunogenic peptides to MHC class I and II antigens. Genetic vaccines unlike attenuated viral vaccines only express a single viral antigen, which reduces the response breadth.

COVID-19 vaccines that have been licensed for emergency use thus far all express spike protein [2–6]. Pre-clinically they induced T and B cells and protection against disease [22–25]. Early clinical trials demonstrated safety, which was confirmed after vaccination campaigns had reached millions of humans causing very rarely serious adverse events such as anaphylactic reactions after the RNA vaccines [26] or the thrombocytopenia with thrombosis syndrome in recipients of some of the Ad vector vaccines [27]. The early trials reported induction of T and B cell responses. Phase III trials confirmed that vaccines were highly efficacious in preventing serious disease or death [2–6]. Ad vector vaccines have advantages over RNA vaccine; they are less costly and more heat stable. One potential disadvantage is that neutralizing antibodies to Ad vectors that are either present in humans due to natural infections or that are induced by vaccination may blunt vaccine immunogenicity [28, 29]. Regarding efficacy of the different Ad vector vaccines, the Johnson and Johnson vaccine that uses a single dose of HAdV-26 showed 66.3% efficacy against infection [30]. Sputnik V, which is a two-dose vaccine that uses an HAdV-26 vector prime followed 4 weeks later by a boost with an HAdV-5 vector reported 91.6% efficacy [6], while the AstraZeneca vaccine, which is based on an AdC vector used in a homologous prime-boost regimen prevents symptomatic COVID-19 disease in 76% of the vaccine recipients [5].

Correlates of protection against COVID-19 remain ill-defined. A role for neutralizing antibodies is supported by protection of nonhuman primates upon adoptive transfer of antibodies [31-33]. Pre-clinical results were confirmed in humans [34] and included trials, which showed that infusion of SARS-CoV-2-specific monoclonal antibodies benefits patients with mild to moderate COVID-19 disease [35]. Antibody-mediated resistance to SARS-CoV-2 infection is likely to be dampened over time by mutations of spike's receptor-binding domain as already suggested pre-clinically by loss of protection of mice that received antisera against an early US isolate prior to challenge with variants from the UK or South Africa [36].

A study that observed breakthrough infections in SARS-CoV-2-immune rhesus macaques upon $CD8^+$ T cell depletion [31] showed that these cells contribute to protection upon a natural infection and presumably upon vaccination. This is supported further by the finding that humans, who are in general protected against a reinfection upon mild or asymptomatic COVID-19 commonly have robust T cells responses in the absence of detectable antibodies [10]. Furthermore, protection against disease upon SARS-CioV-2 infection has been linked to robust anti-viral T cell responses [37, 38].

We developed a set of AdC vaccines that are suited for heterologous prime-boost vaccination. The vaccine carriers, AdC6 and AdC7, represent distinct serotypes so that antibodies induced by one vector fail to neutralize the other, which is also one of the potential advantages of the Sputnik V over the AstraZeneca vaccine. In addition, many humans have robust titers of pre-existing neutralizing antibodies to human Ad serotypes such as HAdV-5 and HAdV-26 [28], which by blocking cell transduction and production of the SARS-CoV-2 antigen by the vaccine may blunt responses. In contrast, neutralizing antibodies to AdC viruses are rare in humans and those who carry them in general have low titers [28, 29]. We selected N as our vaccine antigen assuming that over time it would be less variable than spike, which with growing numbers of SARS-CoV-2-immune humans will increasingly face neutralizing antibody-mediated selection pressure. Furthermore, N has already been shown experimentally to induce T cell responses in naturally infected humans [39, 40] and responses cross-react with other human coronaviruses.

T cells are primarily directed against a small set of so-called immunodominant epitopes within a viral protein and viruses can evade such responses by mutational escape as was shown in chronic infections with for example hepatitis C virus [41] or human immunodeficiency virus [42]. To broaden $CD8^+$ T cell responses one set of AdC vectors expresses the N protein within HSV-1 gD, which by blocking the early BTLA-HVEM checkpoint promotes activation of T cells to subdominant epitopes [17]. As shown by our data, N presented in its wild-type form by either the AdC6 or AdC7 vectors induced after a single immunization very focused $CD8^+$ T cell responses to only one epitope carried by peptide 44 while the gDN fusion protein vaccines elicited responses to several other epitopes. Surprisingly, $CD8^+$ and $CD4^+$ T cell responses to the gDN- but not the N-expressing vectors increased over time so that by 3 months after a single vaccine dose both breadth and magnitude of responses were markedly higher than after 2 weeks. $CD4^+$ T cell responses when tested at 2 weeks after immunization were low but like $CD8^+$ T cell responses increased and gained breadth over time. As a rule, T cell responses peak within 1-2 weeks after exposure to antigen and then, once the antigen has been removed, most effector cells die and responses contract [43]. Ad vectors like Ad viruses tend to persist at low levels and maintain effector T cell responses for prolonged periods of time while T cell transitioning into memory is delayed [44, 45]. Nevertheless, this does not fully explain the unusual response kinetics to the AdC-gDN vaccines. Results from previous experiments that use AdC vectors expressing a different antigen within gD suggest that the checkpoint inhibitor may in part play a role in delaying peak responses [17, 45]. It remains to be investigated if characteristics of N, which is known to allow SARS-CoV-2 to evade innate sensors [46] and, like SARS-CoV-1, may affect IFN pathways [47], also contribute.

Booster immunization with the AdC-N vectors increased the breadth of the $CD8^+$ T cell responses to several additional epitopes but was relatively ineffective for AdC-gDN vectors. Prime-boosting with high doses of the AdC-gDN vectors decreased responses while lower doses performed better potentially indicating that excessive doses of the vaccines cause prolonged activation of T cells rendering them susceptible to activation-induced cell death upon re-exposure to antigen. These results are reminiscent of those obtained in clinical trials with the AstraZeneca vaccine, which showed higher efficacy at a lower vaccine dose [48] and if the booster immunization was given at 3 months rather than within 6 weeks after priming [49].

The vaccine regimens we explored differed in timing of the boost or the analyses. Our results do not suggest that the former plays a major role while the latter clearly allows for heightened and broadened $CD8^+$ but not necessarily $CD4^+$ T cell responses. Lack of a more robust booster response may reflect the previously described delay in T cell memory formation due to vector persistence [44, 45] furthermore suggesting that a single dose regimen may suffice for further clinical development of Ad vector-based T cell vaccines.

The N sequences that were recognized by the vaccine-induced $CD8^+$ and $CD4^+$ T cells were highly conserved within SARS-CoV-2 isolates from around the globe and most showed less than 5% variability. One clear exception was amino acid sequence 221-235 present in peptide 45 (LLLLDRLNQLESKMS) which showed 32.5% and 22.3% variability within North American and European isolates, respectively. Most of the mutations involved AA 234 and 235, which do not appear to play a role in binding to common HLAs suggesting that viral fitness rather than pressure from cell-mediated immunity may have been the cause for selection of these mutants. One study reported selection pressure on amino acid 232 of SARS-CoV-2 N [50]; this AA shows 0.84% variability in our sequence panel but again according to epitope predication is unlikely to be part of a dominant $CD8^+$ T cell epitope. The same paper reported that the AA in position 13 of N, which in the original isolate is a proline, showed the highest degree of variability. Using our sequence panel, we also observed that this AA was frequently exchanged mainly by a leucine. Mutations were more common in the Americas and Asia (> 4.5% of all sequences) but comparatively rare in Oceania (1.2% of all sequences). The proline is part of an epitope with good binding to HLA-B*07:02, which is lost upon its replacement with leucine implying that this mutation may have been caused by T cell-mediated selection pressure. HLA-B*07:02 is rare in the Americas but frequent in the UK (http://igdawg.org/software/browser-beta.html) suggesting that the initial selection may have taken place prior to spread of the variant into the Americas (https://www.cdc.gov/coronavirus/2019-ncov/variants/variant-cases.html).

CONCLUSION

In summary, AdC vector expressing the N protein within HSV-1 gD induce robust and sustained T cell responses. They also induce very broad responses to multiple epitopes within the protein, which is essential to prevent viral escape through mutations. Combined with vectors expressing the viral spike protein for induction of neutralizing antibodies they may prolong vaccine-induced protection and guard against mutational escape, which is especially critical for less developed countries, where access to vaccines is limited.

ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

MH, *RA*, *AC* carried out animal handling, bleeding, prime-boost vaccination; *MH*, *RA* performed intracellular cytokine staining, preparation of splenocytes, *in vitro* stimulation of lymphocytes; *MH* performed RMA-S assay; *MH and MK* conceived flow cytometry experiments, *MH, MK, RA* performed flow cytometry experiments; *MN* performed Western blot analysis, prepared the gel for mass spectrometry, conceived and analyzed conservation of sequences of SARS-CoV-2 isolates; *DN* performed gel ligation for AdC6 and AdC7 clones, linearized clones, and transfected clones into HEK 293 cells, expanded virus, analyzed genetic integrity and stability of vector genome by gel electrophoresis, performed RT-PCR; *XZ* and *DN* performed cloning of vectors and QC; *XZ* designed the viral vectors for the vaccine; *MH, MN, XZ, HE* contributed to the writing and editing the manuscript; *HE* conceptualized, designed, and supervised the project, and analyzed the data. All authors were involved in reviewing the data and manuscript.

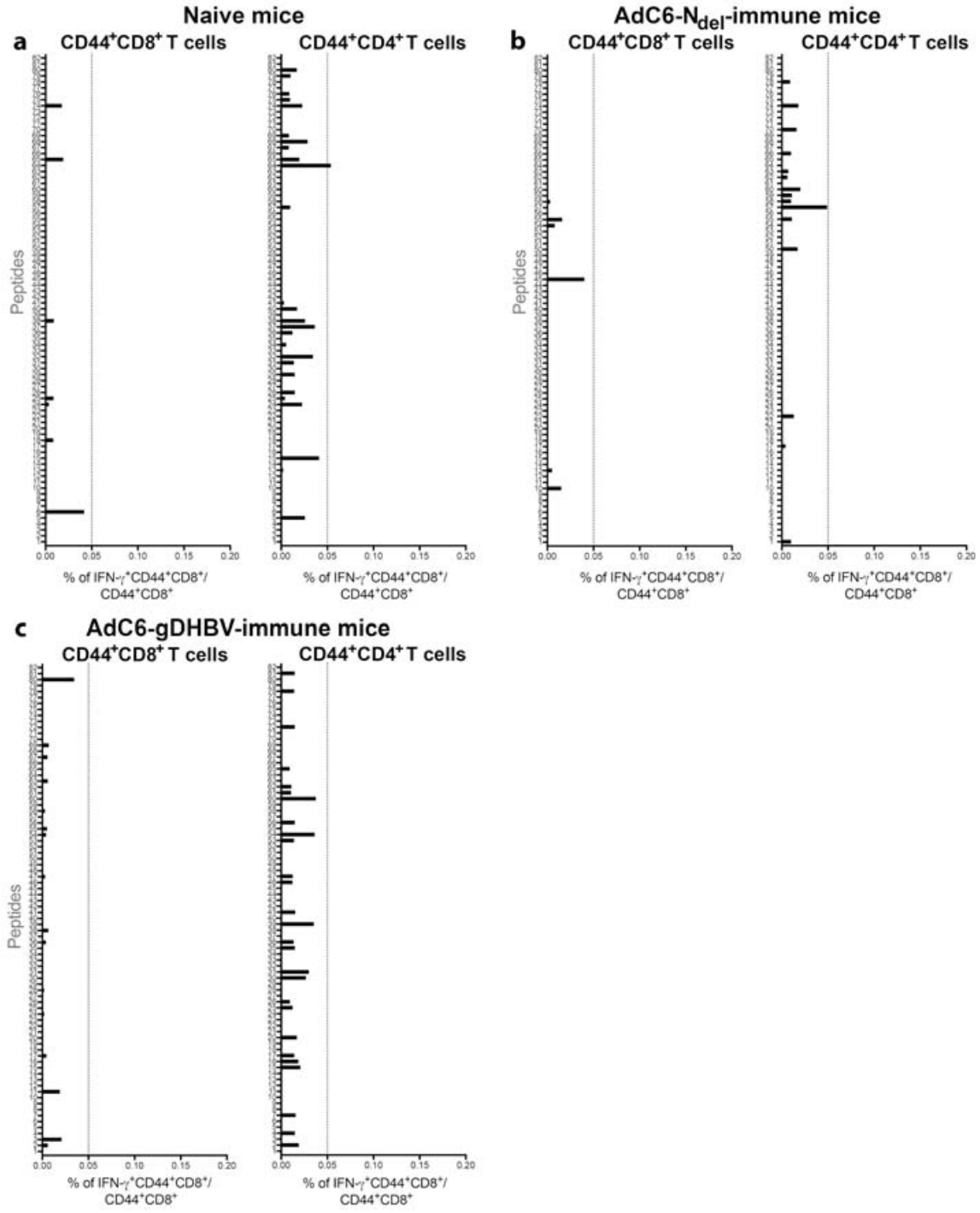
CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

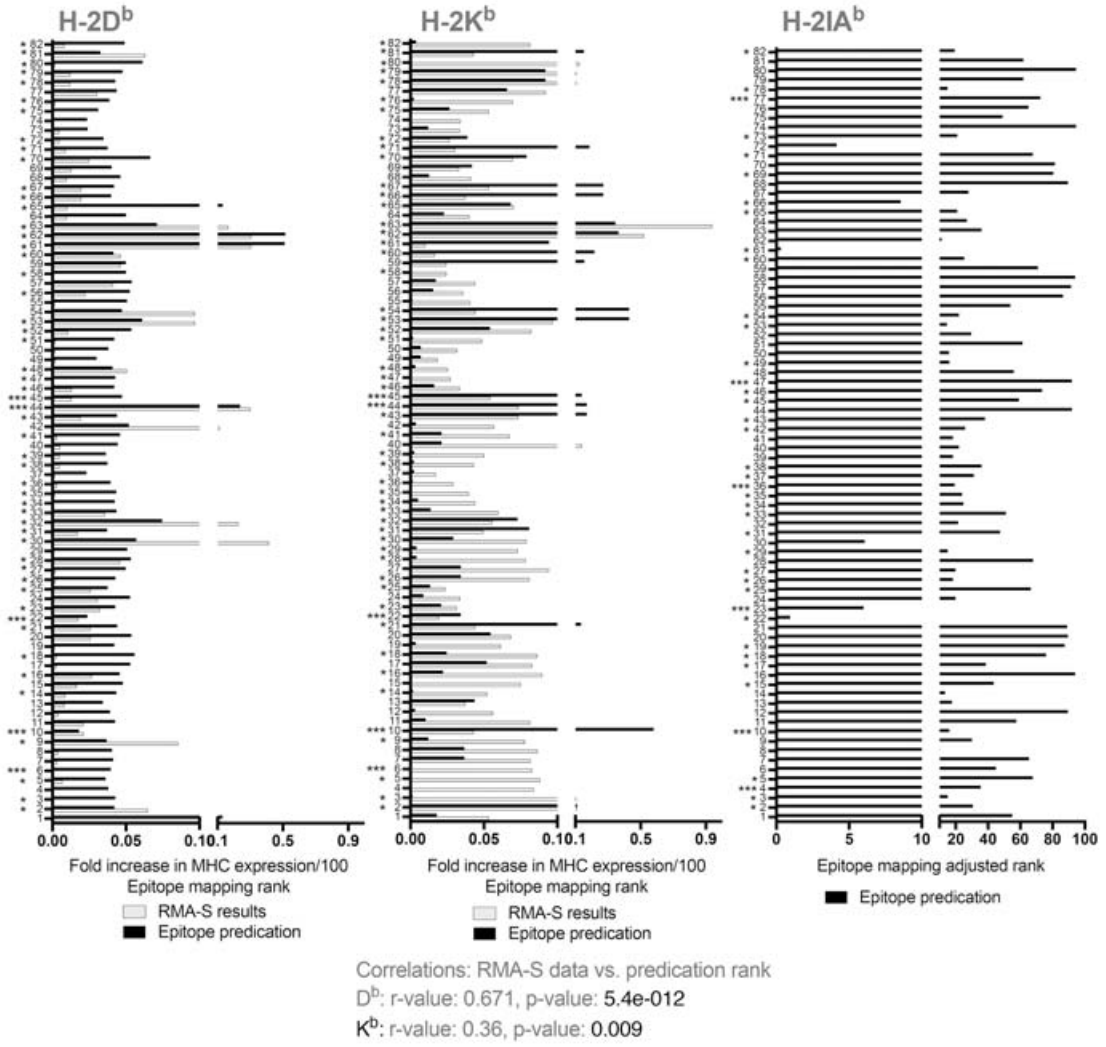
Supplementary Table 1. Amino acid sequence of SARS-CoV-2 N.

1	2	3	4	5	6	7	8	9	10
MSDNGPQNQRNAPRITFGGPSDSTGSNQNGERSGARSKQRRPQGLPNNTA									
11	12	13	14	15	16	17	18	19	20
SWFTALTQHGKEDLKFPRGQGVPINTNSSPDDQIGYYRRATRRIRGGDGK									
21	22	23	24	25	26	27	28	29	30
MKDLSPRWYFYYLGTGPEAGLPYGANKDGIIWVATEGALNTPKDHIGTRN									
31	32	33	34	35	36	37	38	39	40
PANNAAIVLQLPQGTTLPKGFYAEGSRGGSQASSRSSSRSRNSSRNSTPG									
41	42	43	44	45	46	47	48	49	50
SNRGTSPARMAGNGGDAALALLLLDRLNQLESKMSGKGQQQQGQTVTKKS									
51	52	53	54	55	56	57	58	59	60
AAEASKKPRQKRTATKAYNVTQAFGRRGPEQTQGNFGDQELIRQGTDYKH									
61	62	63	64	65	66	67	68	69	70
WPQIAQFAPSASAFFGMSRIGMEVTPSGTWLTYTGAIKLDDKDPNFKDQV									
71	72	73	74	75	76	77	78	79	80
ILLNKHIDAYKTFPPTEPKKDKKKKADETQALPQRQKKQQTVTLLPAADL									
81	82								
DDFSKQLQQSMSSADSTQA									

*Numbers above indicate the 1st amino acids of each 15mer peptide



Supplemental Fig. 1. Recognition of N peptides by naïve T cells directed to irrelevant antigens or T cells to control AdC or AdC-gD vectors. Splenocytes from naïve mice [a], mice immunized with, AdC6-$N_{1\text{-}233}$ or AdC6-$N_{235\text{-}420}$ [b], or an AdC6 vector expressing gD fused to an antigen of hepatitis B virus [c] were stimulated with all of the N peptides [a ,b ,c] or the peptides not present within the sequence expressed by the Ad vectors [c] for 5 hrs *in vitro* and then stained for surface T cell markers and intracellular IFN-γ. Graphs show frequencies of $CD44^+CD8^+$ T cells and $CD44^+CD4^+$ T cells producing the cytokine over all $CD44^+$ T cells within each subset. The line at 0.05% indicated that only one peptide scored positive above this limit for splenocytes of naïve mice.



Supplemental Fig. 2. T cell epitopes defined by predication algorism and MHC class I binding to RMA-S cells. Increase of MHC class I D^b [left graph] or K^b [middle graph] upon incubation with N peptides is shown as grey bars. The bars show fold increases (divided by 100) of MHC class I expression upon incubation of cells with peptides as compared to cells incubated with medium. The rank obtained by epitope prediction is shown as black bars. R- and p-values for Pearson correlation are shown below the graphs. For MHC class II the adjusted rank is shown for each peptide [right graph].

Supplementary Table 2. Predictions for peptide binding to different types of HLA.

Peptide	Amino Acids	Peptide sequence	Binding to HLA*			
			Allele**	Core***	Score	Percentile Rank
2	6–20	PQNQRNAPRITFGGP	HLA-B*15:01	NQRNAPITF	0.67	0.16
9	41-55	RPQGLPNNTASWFTA	HLA-B*57:01	QGLPNNTSW	0.77	0.24
10	46-60	PNNTASWFTALTQHG	HLA-A*68:02	NTASWFTAL	0.87	0.03
21	101-115	MKDLSPRWYFYYLGT	HLA-B*07:02	SPRWYFYYL	0.85	0.07
30	146-160	IGTRNPANNAAIVLQ	HLA-A*30:01	GTRNPANNA	0.57	0.09
31	151-165	PANNAAIVLQLPQGT	HLA-A*68:02	NNAAIVLQL	0.24	0.55

Supplementary Table 2 continued..

32	156-170	AIVLQLPQGTTLPKG	HLA-B*15:01	LQLPQGTTL	0.79	0.07
44	216-230	DAALALLLLDRLNQL	HLA-A*02:01	LLLDRLNQL	0.96	0.02
45	221-236	LLLLDRLNQLESKMS	HLA-A*02:01	LLLDRLNQL	0.96	0.02
61	301-315	WPQIAQFAPSASAFF	HLA-B*15:01	AQFPSASAF	0.99	0.01
63	311-326	ASAFFGMSRIGMEVT	HLA-A*11:01	ASAFFGMSR	0.80	0.08
66	326-340	PSGTWLTYTGAIKLD				
67	331-345	LTYTGAIKLDDKDPN				
70	346-360	FKDQVILLNKHIDAY	HLA-B*15:01	LLNKHIDAY	0.87	0.03
71	351-365	ILLNKHIDAYKTFPP	HLA-B*15:01	LLNKHIDAY	0.87	0.03
81	401-415	DDFSKQLQQSMSSAD				
Peptide	**Amino Acids**	**Peptide sequence**	**Allele**	**Core**	**Score**	**Adjusted Rank**
3	11-25	NAPRITFGGPSDSTG				
22	106-120	PRWYFYYLGTGPEAG	HLA-DRB1*04:05	PRWYFYYLGTGPEA	0.77	0.83
23	111-125	YYLGTGPEAGLPYGA				
61	301-315	WPQIAQFAPSASAFF	HLA-DRB1*09:01	PQIAQFAPSASAFF	0.01	0.01
66	326-340	PSGTWLTYTGAIKLD	HLA-DRB1*07:01	PSGTWLTYTGAIKL	0.40	0.43
82	406-420	QLQQSMSSADSTQA	HLA-DRB1*07:01	PSGTWLTYTGAIKL	0.40	0.43

*Potential binding to common HLAs was determined using an epitope prediction tool (http://tools.iedb.org/main/)
**HLA allele that scored the highest binding
*** Peptide recognized by the corresponding HLA

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