The induction of SARS-CoV-2-specific CD8+ T cell immunity uncouples with the viral spread in K18-hACE2 infected mice

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Article

Keywords:
The induction of SARS-CoV-2-specific CD8⁺ T cell immunity uncouples with the viral spread in K18-hACE2 infected mice

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Abstract

Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV)-2 enters the host by infecting nasal ciliated cells. The virus then spreads toward the oropharyngeal cavity, and, in most severe cases, the pulmonary tissues. The antiviral adaptive response is promptly induced in response to virus detection, with virus-specific T lymphocytes appearing before antiviral antibodies. Both breadth and potency of the antiviral CD8+ T cell immunity have a key role in containing viral spread and disease severity. However, how much the spread of infecting SARS-CoV-2 into the respiratory tracts influences the potency of the antiviral CD8+ T-cell immunity is still largely unknown. To fill the gap, we checked the CD8+ T cell immunity induced after infection of K18-hACE2 transgenic mice in conditions where the virus spread is impeded. In detail, mice were infected with SARS-CoV-2 both 3 weeks and 3 months after anti-Spike vaccinations, and virus-specific CD8+ T cell immunity was monitored both before and after infection. We noticed a strong increase of the Spike-specific CD8+ T cell immunity in vaccinated mice six days after infection despite a nearly-full inhibition of the viral replication. Most important, both kinetics and efficiency of the induction of SARS-CoV-2 N- and M-specific CD8+ T cell immunity in vaccinated mice appeared not inferior to those induced in control mice. These results support the idea that the SARS-CoV-2 replication in the lungs does not relevantly influence the generation of virus-specific CD8+ T cell immunity.

Introduction

Studies on both animals and humans demonstrated that the CD8+ T-cell immunity plays a key role in the recovery from Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV)-2 infection1. Its efficacy is basically unaffected by the amino acid substitutions occurring in emerging viral variants2. Yet, due to the cross-reactivity of virus-specific CD8+ T cells, the infection with low pathogenic Coronaviruses can generate an effective anti-SARS-CoV-2 CD8+ T cell immunity3.

In detail, the role of the antiviral CD8+ T cell immunity in controlling the SARS-CoV-2 infection was for instance highlighted by a study in rhesus macaques which demonstrated that the depletion of CD8+ T cells after a first virus challenge abolished the protective effect of natural immunity against a virus re-challenge carried out after the waning of neutralizing antibodies4. In humans, it was found that a SARS-CoV-2 N-specific CD8+ T cell immunodominant epitope (i.e., HLA B07-restricted N105-113) is associated with the development of a mild disease5. Moreover, oncologic patients with significant impairment of B cells in the presence of preserved CD8+ T-cell counts, showed lower viral loads and reduced mortality upon SARS-CoV-2 infection compared to what observed in homologous patients with low CD8+ T-cell counts, and the depletion of B cells in patients with hematologic cancers did not associate with increased COVID-19-related mortality6.

Concerning the mechanisms underlying the generation of the SARS-CoV-2-specific CD8+ T cell immunity, little is known about the role played by viral spread in the respiratory tracts. The clarification of this point would be of interest for a better comprehension of the dynamics of induction of cellular immune
responses upon natural infection. We tried to shed light on this issue through the analysis of CD8+ T cell immune response soon after infection in K18-hACE2 mice. SARS-CoV-2 replicates in these animals by virtue of the expression in epithelial cells of the human receptor of SARS-CoV-2 virus, i.e., the human angiotensin-converting enzyme (hACE)-2, under the control of the cytokeratin-18 promoter\(^7\). The intranasal infection of K18-hACE2 mice with SARS-CoV-2 led to sustained viral replication in both nasal turbinate and lungs as early as 24 hours post-infection\(^8\). On the basis of this evidence, it could be expected that the prompt diffusion of the infecting virus implies the involvement of lymph nodes and lung germinal centers of different districts of both higher and lower respiratory tracts which could additively contribute to the overall virus-specific CD8+ T-cell immune response. Surprisingly enough, however, we found that the induction of the antiviral CD8+ T cell immunity as detectable in circulation is not influenced by the virus replication extents in the lungs. These data would be of help in understanding the mechanisms underlying the generation of SARS-CoV-2 CD8+ T-cell immunity in asymptomatic subjects.

**Results**

**Induction of SARS-CoV-2-specific CD8+ T cell immunity in infected mice shortly after vaccine boosting.** The role of SARS-CoV-2 spread in the generation of virus-specific CD8+ T cells was evaluated by inhibiting the virus replication in infected K18-huACE2 mice by anti-Spike vaccination. Mice (6 per group) were injected with either Spike-expressing or unrelated DNA vectors twice 2 weeks apart. After additional two weeks, anti-Spike Abs in plasma were measured by ELISA, and PBMCs were tested for the generation of Spike-specific CD8+ T cells by EliSpot assay. Immunized mice showed a strong response in terms of both induction of anti-Spike antibodies (Fig. 1a) and Spike-specific CD8+ T-cells (Fig. 1b). One week later, mice were intranasally infected with 4.4×10^4 TCID\(_{50}\) of SARS-CoV-2, and the viral replication extent in the lungs was assessed 4-6 days after challenge. Viral spread in the lungs appeared dramatically hampered in vaccinated mice, with a mean reduction of more than 5-logs (Fig. 1c), in the absence of weight loss (Supplementary fig. 1). PBMCs from infected mice were isolated either at 4 or 6 days after infection, and the presence of SARS-CoV-2 Spike-, M-, and N-specific CD8+ T cells was evaluated by EliSpot analysis. At day 4 after infection, Spike-specific CD8+ T cells were detectable in vaccinated mice only, while both M- and N- specific CD8+ T cells remained undetectable (Supplementary fig. 2). At day 6 post-infection, the sustained Spike-specific CD8+ T cell immune response in vaccinated mice coupled with the detection of both M- and N- CD8+ T cells at extents not lower than those detected in control mice (Fig. 1d).

We concluded that, shortly after boosting, the extents of virus-specific CD8+ T cell immune response as detected in circulatory cells were not influenced by the viral spread in the lungs.

**Persistent Spike-specific CD8+ T cell immunity and block of virus replication in vaccinated mice three months after boosting.** Electroporation procedures have the potential to induce a both localized and transient immune activation\(^9\). Hence, it was mandatory to evaluate possible influences of both the timing of
infection after boosting and the method of vaccination on the prompt generation of the virus-specific CD8+ T cell immune response we observed in vaccinated/infected mice. To this aim, the immunization experiment was reproduced carrying out both infections and immunologic analyses three months after boosting, and by immunizing mice also with the mRNA-based, commercial Spikevax vaccine.

Mice (10 per group) were immunized with either the Spikevax vaccine or the Spike-expressing DNA vector, and the levels of anti-Spike antibodies were evaluated by ELISA assays both two weeks and three months after boosting (Fig. 2a). Both vaccines proved able to induce high levels of anti-Spike antibodies, with only a partial waning over time. Circulatory Spike-specific CD8+ T cells were assessed by EliSpot/ICS-flow cytometry three months after boosting before virus challenge (Fig. 2b-c). We noticed that the Spike-specific cell immune response persisted, with more sustained values appearing in DNA-vaccinated mice. Mice were then infected as above described, and the SARS-CoV-2 replication extents were evaluated by RT-qPCR carried out on total RNA extracted from lungs 4-6 days after infection. We noticed a more than 5-log reduction of the virus replication in both Spikevax- and DNA-immunized mice (Fig. 3). No weight loss was observed in vaccinated/infected mice (Supplementary fig. 3).

Hence, the immunization with Spikevax vaccine and Spike-expressing DNA in K18-hACE2 mice were effective enough to impede the viral spread also when the infection was carried out three months after vaccine boosting.

SARS-CoV-2 M-specific CD8+ T cells are induced at similar extents after infection of unvaccinated and vaccinated mice three months after boosting. Both EliSpot and ICS-flow cytometry analyses served to monitor the levels of both Spike- and M-specific CD8+ T cells in PBMCs isolated at days 4 or 6 after the here above described infections. At the day 4 post-infection, M-specific CD8+ T cell lymphocytes were undetectable in all infected mice (Supplementary fig. 4), in the presence of the expected Spike-specific CD8+ T cell immunity in vaccinees (Fig. 4). Six days after infection, the percentages of Spike-specific CD8+ T cells increased significantly in both groups compared to day 4 after infection (Fig. 5a-b). Most notably, by that time M-specific CD8+ T cells became readily detectable at similar levels in vaccinated and unvaccinated mice (Fig. 5c).

These results further supported the idea that the bulk of virus-specific CD8+ T cell immune response after infection can be induced independently of the virus spread in the lungs.

Discussion

We sought to establish whether the spread of SARS-CoV-2 contributes to the induction of viral-specific CD8+ T cell immune response. Shedding light in such a still unexplored issue would be of utility for the interpretation of the dynamics of the induction of CD8+ T cell immunity in infected people, as well as for the development of new mucosal CD8+ T cell- based anti-SARS-CoV-2 vaccines.

Upon intranasal infection of K18-hACE2 mice, the virus diffuses quite rapidly, so that similar amounts of infectious virus were found in both nasal turbinate and lungs as early as 24 hours post
The viral load gradually decreases in nasal turbinate, while it peaks at day 2 post infection in the lungs. Thereafter, the viral load in the lungs decreases, however persisting at high levels until days 6-7 post infection. The vaccination protocols we applied appeared quite effective in reducing the viral load in infected mice. The residual viral RNA detected in vaccinated mice could be consequence of very low levels of actual virus replication, or it may be residual viral RNA accumulated in abortively infected cells/defective viral particles. In any case, both DNA and Spikevax vaccines reduced by more than 5 logs the viral load, as measured in the lungs. When we investigated whether such a reduction reflected the overall induction of circulatory SARS-CoV-2-specific CD8+ T cells, we did not find major differences between control and vaccinated mice apart from, as expected, the levels of Spike-specific CD8+ T lymphocytes which appeared boosted in vaccinated mice. De novo SARS-CoV-2-specific circulatory CD8+ T cells appeared not before 6 days after challenge irrespectively of the vaccination status.

The overall levels of Spike-specific immune responses appeared stronger in mice vaccinated with DNA compared to those receiving the Spikevax vaccine. Most probably, this effect was the consequence of higher nucleic acid loading in DNA vaccinated mice, i.e., 10 micrograms/leg, which was followed by the electroporation protocol to optimize the DNA cell entry, compared to the injection of the equivalent of 0.5 micrograms/leg of Spike-specific mRNA in Spikevax vaccinated mice.

Data from the experiment carried out three months after vaccine boosting excluded possible biases due to a general transient immune hyper-activation consequence of electroporation procedures, and/or DNA loading. Additional confounding effects not related to Spike-specific vaccination can be also excluded considering the similar results obtained in Spikevax vaccinated mice. Taken together, the presented data strongly support the idea that efficient viral spread in the respiratory tracts is not necessary to generate an optimal CD8+ T cell-specific immune response.

Our study has several limitations. The experimental groups included a limited number of mice; viral replication was evaluated in the lungs only. However, we referred to the already demonstrated evidence that the viral replication was inhibited at similar extents in the lungs, turbinate, and oral cavity of vaccinated mice. Additional limitations include the lack of investigation on both phenotype and function of the virus-specific CD8+ T cells, and the tissue-specific disposition of the virus-specific CD8+ T cells induced after infection. On this subject, however, it was reported that the frequency of tissue-resident SARS-CoV-2-specific T lymphocytes correlated with that of circulatory ones.

However, our data consistently demonstrated that the induction of virus-specific CD8+ T cell immunity does not require high levels of viral spread. Translating this finding in humans, one can assume that a similar virus-specific CD8+ T cell immune response can take place in asymptomatic/mildly affected subjects and in those recognizing a severe disease due to the spread of the virus in the lungs. Considering the long-lived CD8+ T cells found in SARS-CoV survivors (till 17 years), as well as the key role of the CD8+ T cell immunity in the protection from SARS-CoV-2 infection, it is tempting to speculate that the asymptomatic/mild infection has the potential to act as a natural, long-lasting immunization that may induce a sort of CD8+ T cell-based herd immunity. Searching for SARS-CoV-2-specific CD8+ T cells in PBMCs...
isolated from people not experiencing Covid-19 symptoms would be of utility to confirm this hypothesis. On this subject, 4 out of 11 asymptomatic individuals testing negative at the RT-PCR assay but having close contact with infected people showed SARS-CoV-2 specific CD4+ and CD8+ T-cell immune responses\(^{21}\).

On the other hand, our data suggest the feasibility of innovative vaccines focused on the induction of a systemic antiviral CD8+ T cell immunity by mucosal administration of inactivated viral particles, SARS-CoV-2-based VLPs, or nanovesicles associating SARS-CoV-2 products.

**Materials & Methods**

**Animals and authorizations**

Six-week-old, female C57 Bl/6 K18-hACE2 transgenic mice were purchased from Charles River (Calco, Italy) and hosted at the Central and BSL3 Animal Facilities of the Istituto Superiore di Sanità. The study was conducted according to the guidelines of the Declaration of Helsinki, and approved as approved by the Italian Ministry of Health, authorization 591/2021, released on July 30th 2021. Authors complied with the ARRIVE guidelines, included the use of control mice shared with already reported heterologous experiments\(^{22}\). Before the first procedure, Datamars (Lugano, Switzerland) microchips were inserted subcutaneously on the dorsal midline between the shoulder blades.

**Anti-Spike vaccines**

The Ph-CMV DNA vector expressing full-length, codon optimized wild type SARS-CoV-2 Spike protein open reading frame (ORF) (Wuhan-Hu-1, GenBank: NC_045512.2) was a gift from A. Cara\(^{23}\). Mice were immunized also with mRNA-based anti-Spike Moderna Spikevax vaccine, lot # 000030A, 0.2 mg/mL.

**Mice immunization**

Isoflurane-anesthetized mice were inoculated i.m. with 10 \(\mu\)g of DNA in 30 \(\mu\)L of sterile, 0.9% saline solution. DNA injection was immediately followed by electroporation at the site of inoculation with an Agilpulse BTX (Holliston, MA) device, using a 4-needle electrode array (4 mm gap, 5 mm needle length), and applying the following parameters: 1 pulse of 450 V for 50 \(\mu\)s; 0.2 ms interval; 1 pulse of 450 V for 50 \(\mu\)s; 50 ms interval; 8 pulses of 110 V for 10 ms with 20 ms intervals. Mice in the Spikevax-immunization group were first mock-inoculated with 30 \(\mu\)L of sterile, 0.9% saline solution followed by electroporation as described above, and then vaccinated by i.m. injection in a different site (out of the electroporated area) with 30 \(\mu\)L of Spikevax containing 0.5 \(\mu\)g of RNA. Mice were immunized into both quadriceps, twice, 2 weeks apart. Mice were sacrificed by cervical dislocation.
Anti-Spike ELISA

SARS-CoV-2 recombinant S1 protein (Sigma AGX 818) was used for coating 96 well plates (Greiner bio-one, Frickenhausen, Germany) with 0.1 µg/well of protein overnight at 4°C. Plates were washed and blocked with 1% BSA (Sigma Chemicals) in PBS. Duplicate wells of 2-fold serial dilutions of plasma from individual mice were incubated for 2 h at room temperature (RT). After washing, horse radish peroxidase (HRP)-goat anti-mouse immunoglobulin (Invitrogen, cat. # 31430) was added to plates and incubated for 2 h at RT. Plates were therefore washed and incubated with 100 µL/well of 3.3,5,5-tetramethylbenzidine substrate (SurModics BioFX, Edina, MN) for 8 minutes at RT. The reaction was blocked with 50 µL/well of 1 M H2SO4. Endpoint titers were calculated as the reciprocal of the highest dilution that exceeded the cut-off, calculated as the mean absorbance of plasma from naïve mice + 0.1 O.D.

IFN-γ EliSpot analysis

Peripheral blood mononuclear cells (PBMCs) were recovered from EDTA-blood samples obtained through retro-orbital puncture under topical anesthesia. Erythrocytes were removed by treatment with ACK lysing buffer (Gibco) according to the manufacturer’s instructions.

A total of 10^5 live cells were seeded in triplicate in microwells of 96-multiwell plates (Millipore, Burlington, MA) previously coated with the anti-mouse IFN-γ AN18 mAb (Mabtech, Nacka Strand, Sweden) in RPMI 1640, 10% heat-inactivated fetal calf serum (FCS, Gibco, Thermo Fisher), and 50 µM 2-mercaptoethanol. Cell cultures were carried out for 16 h in the presence of 5 µg/mL of the following CD8-specific, H2b-binding, SARS-CoV-2-specific peptides: Spike539–546: VNFNFNGL, M173-180: RTLSYYKL, and N219–228: ALALLLDRL. As negative controls, 5 µg/mL of unrelated H2b binding peptides were used. Peptide preparations were obtained from BEI resources. To check for cell responsiveness, 10 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma, St. Louis, MO) plus 500 ng/mL of ionomycin (Sigma) were added to the cultures. After 16 h, the cells were discarded, and the plate was incubated for 2 h at room temperature with R4-6A2 biotinylated anti-IFN-γ antibody (Mabtech) at the concentration of 100 μg/mL. Wells were then washed and treated for 1 h at room temperature with 1:1,000 diluted streptavidin-ALP from Mabtech. Afterwards, 100 µL/well of SigmaFast BCIP/NBT were added to the wells to develop spots. Spot-forming cells were finally analyzed and counted using an AELVIS EliSpot reader (Hannover, Germany).

Intracellular cytokine staining (ICS) and flow cytometry analysis

Cells collected from blood were cultured at 1×10^7/mL in RPMI medium, 10% FCS, 50 µM 2-mercaptoethanol (Sigma), 1 µg/mL brefeldin A (BD Biosciences, Franklin Lakes, NJ), and in the presence of 5 µg/mL of either Spike or unrelated H2-b CD8+ T-specific peptides. Positive controls were conducted by adding 10 ng/mL PMA (Sigma) plus 1 µg/mL ionomycin (Sigma). After 16 h, cells were stained with 1 µL of LIVE/DEAD Fixable FVD-eFluor506 Dead Cell reagent (Invitrogen Thermo Fisher) in 1 mL of 1xPBS for 30 min at 4 °C, and excess dye removed by 2 washes with 500 µL of 1xPBS. Non-specific staining was minimized by pre-incubating cells with 0.5 µg of Fc blocking mAbs (i.e., anti-CD16/CD32 antibodies,
Invitrogen/eBioscience Thermo Fisher) in 100 µL of 1×PBS with 2% FCS for 15 min at 4 °C. Staining for cell surface markers was performed by incubation for 1 h at 4 °C with 2 µL of the following anti-mouse Abs: FITC-conjugated anti-CD3, APC-Cy7-conjugated anti-CD8a, PerCP-conjugated anti-CD4, and BUV395-conjugated anti-CD44 (BD Biosciences). For intracellular cytokine staining (ICS), cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences), according to the manufacturer’s recommendations. Thereafter, cells were labeled for 1 h at 4 °C with 2 µL of PE-Cy7-conjugated mAb in a total of 100 µL of 1× Perm/Wash Buffer (BD Biosciences). After two washes, cells were fixed in 200 µL of 1× PBS/formaldehyde (2% v/v). Samples were then acquired by a CytoFLEX LX (Beckman Coulter, Brea, CA, USA) flow cytometer and analyzed using Kaluza software (Beckman Coulter). Gating strategy was as follows: live cells as assessed by LIVE/DEAD dye vs. FSC-A, singlet cells from FSC-A vs. FSC-H (singlet 1) and SSC-A vs. SSC-W (singlet 2), CD3+ cells from CD3-FITC vs. SSC-A, CD8+, or CD4+ cells from CD8-APC-Cy7 vs. CD4-PerCP. CD3+/CD8+ cell population was gated against CD44+ cells, and, to detect polyfunctional CD8+ T lymphocytes, the population of cells positive for both CD8 and CD44 was analyzed for PE-Cy7 to detect simultaneous changes in IFN-γ production.

**Virus production**

VERO-E6 cells were grown in DMEM (Gibco, Thermo Fisher) supplemented with 2% FCS, 100 units/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and non-essential amino acids (Gibco). The ancestral SARS-CoV-2/Italy INMI1#52284 viral isolate was propagated by inoculation of 70% confluent VERO-E6 cells. Infected cell culture supernatant was harvested at 72 h post infection, clarified, aliquoted, and stored at -80 °C.

**Mouse infection**

Before experimental infection, mice were anesthetized with a combination of ketamine (50 mg/kg of body weight) and medetomidine (1 mg/kg of body weight) administered intraperitoneally. A volume of 30 µL of each dilution was administered intranasally (i.n.), at 15 µL per nostril, dropwise. After virus challenge, intraperitoneal injection of atipamezole (1 mg/kg of body weight) was used as a reversal agent. In vitro titration of the SARS-CoV-2/Italy INMI1#52284 isolate we used was already described\(^2\). Virus challenge in immunized mice was performed using a virus dose of 4.4 TCID\(_{50}\).

**Extraction and purification of lung RNA**

Lungs were stored frozen after excision. After thawing, equal amounts of tissues were minced and incubated for 10 min in 1 mL of TRIzol™ Reagent (Thermo Fisher Scientific). Minced tissue was then passed through a QIAshredder homogenizer (Qiagen, Germantown, MD), and the flow-through was used for chloroform extraction according to the TRIzol™ protocol, using 0.2 mL of chloroform. Recovered total RNA was stored in water at -80 °C.
**RT-qPCR**

RT-qPCR for SARS-CoV-2 Envelope (E) and Nucleocapsid (N) genes was performed using a One-Step Taqman-based strategy as previously described\textsuperscript{26}. Mouse β-actin amplification was included as loading control. All probes and primers were purchased from Integrated DNA Technologies (IDT). Each 20 μL reaction mixture contained 12 μL of qPCRBIO Probe 1-Step Virus Detect Lo ROX master mix (PCR Biosystems Ltd. London, UK), 3 μL of primers/probes mix, and of 1 μg of ezDNAse (Thermo Fisher)-treated RNA in a total of 5 μL. All samples were tested in duplicate, and samples with nuclease-free water alone were included as negative controls. Serial 10-fold dilutions of E gene plasmid (10006896, 2019-nCoV_E Positive Control from Charité/Berlin, IDT, Leuven, Belgium) and N1/N2 plasmid (10006625, 2019-nCoV_N_Positive Control from CDC, IDT, Belgium) were used to generate standard curves ranging from 1 to $10^5$ copies. Median standard curve slope ranged from 3.25 to 3.43 with $R^2$>0.998.

Samples were run on an Applied Biosystems 7500 Fast PCR system (Thermo Fisher). The following cycling conditions were applied: reverse transcription for 10 min at 55 °C followed by denaturation at 95 °C for 3 min. Then, 50 cycles of denaturation at 95 °C for 15 s and annealing/extension at 58 °C for 30 s. Amplification data were analyzed using Applied Biosystems 7500 software v2.3 (ThermoFisher). Results are reported as numbers of RNA copies for μg of total RNA.

**Statistical Analysis**

When appropriate, data are presented as mean ± standard error (SE). When indicated, the one- or two-tailed Mann–Whitney U test, the two-tailed Student T Test with Welch Correction, or the Kruskal-Wallis Test followed by Dunn’s Multiple Comparisons Test were conducted. $p$< 0.05 was considered significant.

**Data availability**

All data produced during the present study are available upon request made to correspondence author.

**Materials availability**

All requests for reagents should be directed to and will be fulfilled, upon the completion of a Material Transfer Agreement, by the correspondence author.

**References**


13. EMA/896245/2022 Committee for Medicinal Products for Human Use (CHMP) Assessment report Spikevax Procedure No. EMEA/H/C/005791/II/0075/G


**Acknowledgments:** This work was supported by an institutional grant from Istituto Superiore di Sanità, Rome, Italy. Both SARS-Related Coronavirus 2, Isolate Italy-INMI1, NR-52284, and the peptide array of SARS-Related Coronavirus 2 Spike Glycoprotein, NR-52402, were obtained through BEI Resources, NIAID, NIH. We thank Patrizia Cocco, Ferdinando Costa, Daniela Diamanti, Fabiola Diamanti, Daniele Macchia, Enrico Cardarelli, and Pietro Arciero Istituto Superiore di Sanità, for technical support, and Federica Magnani and Rosangela Duranti Istituto Superiore di Sanità for secretarial assistance.


**Competing interests:** The authors declare no competing interests
Legend to figures

**Figure 1.** Spike-specific immune responses and viral replication in DNA injected mice. **a** Detection of anti-S1 antibodies in plasma of K18-hACE2 mice i.m. injected with either void (Ctrl, 6 mice) or Spike-expressing (6 mice) DNA vectors. Shown are the log_{10} of reciprocal endpoint titers, together with intragroup mean ±SE. **b** Detection of SARS-CoV-2-Spike-specific CD8+ T cells in PBMCs. A total of 10^5 PBMCs were incubated overnight with 5 μg/ml of either unrelated or Spike-specific peptide in IFN-γ EliSpot microwells. Shown are the numbers of spot-forming units (SFUs)/well calculated as mean values of triplicates after subtraction of the mean spot numbers detected in wells of PBMCs treated with an unspecific peptide. Reported are intragroup mean values ±SE. **c** Viral loads in lungs of immunized/infected mice. Four and six days after challenge, injected mice (6 per group) were sacrificed, and lungs were processed for the extraction of total RNA. One μg of total RNA from each infected mouse was then analyzed by RT-qPCR for the presence of both SARS-CoV-2 E- and N-specific RNAs. Shown are the means between E and N viral RNA copies amplified from total RNA isolated from lungs of each animal, together with intragroup mean values ±SE. **d** Detection of Spike-, M-, and N-specific CD8+ T cells in PBMCs isolated 6 days after infection. A total of 10^5 PBMCs were incubated overnight with or without 5 μg/ml of either unrelated, Spike-, M-, or N- specific peptides in IFN-γ EliSpot microwells. Shown are the numbers of SFUs/well calculated as mean values of triplicates after subtraction of the mean spot numbers calculated in wells of PBMCs treated with unspecific peptides. Reported are intragroup mean values ±SE.

**Figure 2.** Spike-specific immune responses in both Spikevax and DNA-Spike vaccinated mice. **a** Detection of anti-S1 antibodies in plasma of K18-hACE2 mice two weeks (on the left) and three months (on the right) after injection with either Spikevax or DNA-Spike vaccines. As control, mice were injected with isotonic buffer (Ctrl). Measure were done in plasma from 10 (two weeks after boosting) and 4 (three months after boosting) mice per group. Shown are the log_{10} of reciprocal endpoint titers, together with intragroup means ±SE. **B.** Detection of Spike-specific CD8+ T lymphocytes in cultures of cells isolated from lungs of K18-hACE2 mice three months after vaccination. **b** Detection of SARS-CoV-2-Spike-specific CD8+ T cells in splenocytes isolated from K18-hACE2 mice i.m. injected with either Spikevax vaccine, Spike-expressing DNA vector, or buffer (Ctrl, 4 mice per group) three months after vaccination. A total of 2.5×10^5 splenocytes were incubated overnight with 5 μg/ml of either unrelated or Spike-specific peptide in IFN-γ EliSpot microwells. Shown are the numbers of spot-forming units (SFUs)/well calculated as mean values of triplicates after subtraction of the mean spot numbers detected in wells with splenocytes treated with the unspecific peptide. Reported are intragroup mean values ±SE. **c** Representative results from ICS/flow cytometry analysis for the expression of IFN-γ in PBMCs from mice injected as indicated. Cells were cultivated overnight with either Spike- or an unrelated peptide. Shown are data obtained with PBMCs pooled from two mice per condition representative of two experiments. Quadrants were set on the basis of cell fluorescence of samples treated with the unrelated peptide.
**Figure 3.** Viral loads in the lungs of mice infected three months after boosting. Four to six days after challenge, mice (6 per group) were sacrificed, and lungs processed for the extraction of total RNA. One μg of total RNA from each infected mouse was then analyzed by RT-qPCR for the presence of both SARS-CoV-2 E- and N-specific RNAs. Shown are the means between E- and N-specific viral RNA copies amplified from total RNA isolated from lungs of each animal, together with intragroup mean values ±SE.

**Figure 4.** Detection of Spike-specific CD8⁺ T cells in K18-hACE2 mice three months after vaccine boosting and 4 days after infection. a Data from EliSpot analysis. A total of 10⁵ PBMCs isolated from infected and vaccinated or unvaccinated mice (3 per group) were incubated overnight with 5 μg/ml of either unrelated or Spike-specific peptide in IFN-γ EliSpot microwells. Shown are the numbers of spot-forming units (SFUs)/well calculated as mean values of triplicates after subtraction of the mean spot numbers detected in wells of PBMCs treated with an unspecific peptide. Reported are intragroup mean values ±SE. b Shown are the data from ICS/flow cytometry analysis for the expression of IFN-γ in PBMCs. Cells were cultivated overnight with either Spike or an unrelated peptide. The results obtained with PBMCs pooled from three mice per condition are reported. Quadrants were set on the basis of cell fluorescence of samples treated with an unrelated peptide.

**Figure 5.** Detection of both Spike- and M-specific CD8⁺ T cells in K18-hACE2 mice three months after boosting and 6 days after infection. a Detection of Spike-specific CD8⁺ T cells in both vaccinated and unvaccinated mice (3 per group). Data from EliSpot analysis are shown. A total of 10⁵ PBMCs were incubated overnight with 5 μg/ml of either unrelated or Spike-specific peptide in IFN-γ EliSpot microwells. Shown are the numbers of spot-forming units (SFUs)/well calculated as mean values of triplicates after subtraction of the mean spot numbers detected in wells of PBMCs treated with an unspecific peptide. Reported are intragroup mean values ±SE. b Representative results from ICS/flow cytometry analysis for the expression of IFN-γ in PBMCs are reported. Cells were cultivated overnight with either Spike or an unrelated peptide. Shown are data obtained with PBMCs pooled from three mice per condition. Quadrants were set on the basis of cell fluorescence of samples treated with the unrelated peptide. c Detection of M-specific CD8⁺ T cells in PBMCs isolated 6 days after infection. A total of 10⁵ PBMCs were incubated overnight with or without 5 μg/ml of either unrelated, or M-specific peptides in IFN-γ EliSpot microwells. Shown are the numbers of SFUs/well calculated as mean values of triplicates after subtraction of mean spot numbers calculated in wells of PBMCs treated with unspecific peptides. Intragroup mean values ±SE are reported.
Figures

Figure 1

Spike-specific immune responses and viral replication in DNA injected mice. a Detection of anti-S1 antibodies in plasma of K18-hACE2 mice i.m. injected with either void (Ctrl, 6 mice) or Spike-expressing (6 mice) DNA vectors. Shown are the log10 of reciprocal endpoint titers, together with intragroup mean ±SE.
b Detection of SARS-CoV-2-Spike-specific CD8+ T cells in PBMCs. A total of 105 PBMCs were incubated overnight with 5 μg/ml of either unrelated or Spike-specific peptide in IFN-γ EliSpot microwells. Shown are the numbers of spot-forming units (SFUs)/well calculated as mean values of triplicates after subtraction of the mean spot numbers detected in wells of PBMCs treated with an unspecific peptide. Reported are intragroup mean values ±SE.
c Viral loads in lungs of immunized/infected mice. Four and six days after challenge, injected mice (6 per group) were sacrificed, and lungs were processed for the extraction of total RNA. One μg of total RNA from each infected mouse was then analyzed by RT-qPCR for the presence of both SARS-CoV-2 E- and N-specific RNAs. Shown are the means between E and N viral RNA copies amplified from total RNA isolated from lungs of each animal, together with intragroup mean values ±SE.
d Detection of Spike-, M-, and N-specific CD8+ T cells in PBMCs isolated 6 days after infection. A total of 105 PBMCs were incubated overnight with or without 5 μg/ml of either unrelated, Spike-, M-, or N- specific peptides in IFN-γ EliSpot microwells. Shown are the numbers of SFUs/well calculated as mean values of triplicates after subtraction of the mean spot numbers calculated in wells of PBMCs treated with unspecific peptides. Reported are intragroup mean values ±SE.
Figure 2

Spike-specific immune responses in both Spikevax and DNA-Spike vaccinated mice. a Detection of anti-S1 antibodies in plasma of K18-hACE2 mice two weeks (on the left) and three months (on the right) after injection with either Spikevax or DNA-Spike vaccines. As control, mice were injected with isotonic buffer (Ctrl). Measure were done in plasma from 10 (two weeks after boosting) and 4 (three months after boosting) mice per group. Shown are the log10 of reciprocal endpoint titers, together with intragroup
means ±SE. B. Detection of Spike-specific CD8+ T lymphocytes in cultures of cells isolated from lungs of K18-hACE2 mice three months after vaccination. b Detection of SARS-CoV-2-Spike-specific CD8+ T cells in splenocytes isolated from K18-hACE2 mice i.m. injected with either Spikevax vaccine, Spike-expressing DNA vector, or buffer (Ctrl, 4 mice per group) three months after vaccination. A total of 2.5×105 splenocytes were incubated overnight with 5 μg/ml of either unrelated or Spike-specific peptide in IFN-γ EliSpot microwells. Shown are the numbers of spot-forming units (SFUs)/well calculated as mean values of triplicates after subtraction of the mean spot numbers detected in wells with splenocytes treated with the unspecific peptide. Reported are intragroup mean values ±SE. c Representative results from ICS/flow cytometry analysis for the expression of IFN-γ in PBMCs from mice injected as indicated. Cells were cultivated overnight with either Spike- or an unrelated peptide. Shown are data obtained with PBMCs pooled from two mice per condition representative of two experiments. Quadrants were set on the basis of cell fluorescence of samples treated with the unrelated peptide.
Viral loads in the lungs of mice infected three months after boosting. Four to six days after challenge, mice (6 per group) were sacrificed, and lungs processed for the extraction of total RNA. One μg of total RNA from each infected mouse was then analyzed by RT-qPCR for the presence of both SARS-CoV-2 E- and N-specific RNAs. Shown are the means between E- and N-specific viral RNA copies amplified from total RNA isolated from lungs of each animal, together with intragroup mean values ±SE.
Detection of Spike-specific CD8+ T cells in K18-hACE2 mice three months after vaccine boosting and 4 days after infection. a Data from EliSpot analysis. A total of 105 PBMCs isolated from infected and vaccinated or unvaccinated mice (3 per group) were incubated overnight with 5 μg/ml of either unrelated or Spike-specific peptide in IFN-γ EliSpot microwells. Shown are the numbers of spot-forming units (SFUs)/well calculated as mean values of triplicates after subtraction of the mean spot numbers detected.
in wells of PBMCs treated with an unspecific peptide. Reported are intragroup mean values ± SE. Shown are the data from ICS/flow cytometry analysis for the expression of IFN-γ in PBMCs. Cells were cultivated overnight with either Spike or an unrelated peptide. The results obtained with PBMCs pooled from three mice per condition are reported. Quadrants were set on the basis of cell fluorescence of samples treated with an unrelated peptide.

**Figure 5**
Detection of both Spike- and M-specific CD8+ T cells in K18-hACE2 mice three months after boosting and 6 days after infection. a Detection of Spike-specific CD8+ T cells in both vaccinated and unvaccinated mice (3 per group). Data from EliSpot analysis are shown. A total of 105 PBMCs were incubated overnight with 5 μg/ml of either unrelated or Spike-specific peptide in IFN-γ EliSpot microwells. Shown are the numbers of spot-forming units (SFUs)/well calculated as mean values of triplicates after subtraction of the mean spot numbers detected in wells of PBMCs treated with an unspecific peptide. Reported are intragroup mean values ±SE.
b Representative results from ICS/flow cytometry analysis for the expression of IFN-γ in PBMCs are reported. Cells were cultivated overnight with either Spike or an unrelated peptide. Shown are data obtained with PBMCs pooled from three mice per condition. Quadrants were set on the basis of cell fluorescence of samples treated with the unrelated peptide.
c Detection of M-specific CD8+ T cells in PBMCs isolated 6 days after infection. A total of 105 PBMCs were incubated overnight with or without 5 μg/ml of either unrelated, or M-specific peptides in IFN-γ EliSpot microwells. Shown are the numbers of SFUs/well calculated as mean values of triplicates after subtraction of mean spot numbers calculated in wells of PBMCs treated with unspecific peptides. Intragroup mean values ±SE are reported.

Supplementary Files

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- SupplementaryFigures.pdf