DOI: 10.1002/eji.202149167 Eur. J. Immunol. 2021. 51: 1774–1784

Basic

European Journal of Immunology

Immunity to infection

Research Article

# A single subcutaneous or intranasal immunization with adenovirus-based SARS-CoV-2 vaccine induces robust humoral and cellular immune responses in mice

*Eun Kim*[*1], Florian J. Weisel*[*2], Stephen C. Balmert[3], Muhammad S. Khan[1,4], Shaohua Huang[1], Geza Erdos[3], Thomas W. Kenniston[1], Cara Donahue Carey[3], Stephen M. Joachim[2], Laura J. Conter[2], Nadine M. Weisel[2], Nisreen M. A. Okba[5], Bart L. Haagmans[5], Elena Percivalle[6], Irene Cassaniti[6], Fausto Baldanti[6,7], Emrullah Korkmaz[3,8], Mark J. Shlomchik**[2], Louis D. Falo Jr**[3,8,9,10,11] and Andrea Gambotto**[1,4,11,12,13]*

[1] Department of Surgery, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
[2] Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
[3] Department of Dermatology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
[4] Department of Infectious Diseases and Microbiology, University of Pittsburgh Graduate School of Public Health, Pittsburgh, PA, USA
[5] Department of Viroscience, Erasmus Medical Center Rotterdam, Rotterdam, The Netherlands
[6] Molecular Virology Unit, Microbiology and Virology Department, IRCCS Policlinico San Matteo, Pavia, Italy
[7] Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy
[8] Department of Bioengineering, University of Pittsburgh Swanson School of Engineering, Pittsburgh, PA, USA
[9] Clinical and Translational Science Institute, University of Pittsburgh, Pittsburgh, PA, USA
[10] The McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA, USA
[11] UPMC Hillman Cancer Center, Pittsburgh, PA, USA
[12] Department of Medicine, Division of Infectious Disease, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
[13] Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

**Optimal vaccines are needed for sustained suppression of SARS-CoV-2 and other novel coronaviruses. Here, we developed a recombinant type 5 adenovirus vector encoding the gene for the SARS-CoV-2 S1 subunit antigen (Ad5.SARS-CoV-2-S1) for COVID-19 immunization and evaluated its immunogenicity in mice. A single immunization with Ad5.SARS-CoV-2-S1 via S.C. injection or I.N delivery induced robust antibody and cellular immune responses. Vaccination elicited significant S1-specific IgG, IgG1, and IgG2a endpoint titers as early as 2 weeks, and the induced antibodies were long lasting. I.N. and S.C. administration of Ad5.SARS-CoV-2-S1 produced S1-specific GC B cells in cervical and axillary LNs, respectively. Moreover, I.N. and S.C. immunization evoked significantly greater antigen-specific T-cell responses compared to unimmunized control groups with indications that S.C. injection was more effective than I.N. delivery in eliciting cellular immune responses. Mice vaccinated by either route demonstrated significantly increased virus-specific neutralization antibodies on weeks 8 and 12 compared to control groups, as well as BM antibody forming cells (AFC), indicative of long-term immunity. Thus, this Ad5-vectored SARS-CoV-2 vaccine candidate showed promising immunogenicity following delivery to mice by S.C. and I.N. routes of administration, supporting the further development of Ad-based vaccines against COVID-19 and other infectious diseases for sustainable global immunization programs.**

*Correspondence:* Andrea Gambotto
e-mail: gambottoa@upmc.edu

*Mark J. Shlomchik, Louis D. Falo Jr, and Andrea Gambotto are cosenior authors.
**Eun Kim and Florian J. Weisel equally contributed to this work.
[Correction added on 18 May 2021, after first online publication: URL for peer review history has been corrected.]

**Keywords:** Adenovirus · COVID-19 · Infectious diseases · Recombinant DNA vaccines · SARS-CoV-2

Additional supporting information may be found online in the Supporting Information section at the end of the article.

## Introduction

Outbreaks caused by coronaviruses represent an unprecedented global health challenge. Previous coronavirus outbreaks, Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS), have been a cause of substantial morbidity and mortality [1–3].The ongoing COVID-19 outbreak, caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), constitutes a major threat to public health and global economic growth [4–7]. COVID-19, which first emerged in late 2019, was declared a global pandemic by the World Health Organization on March 11, 2020, and has claimed approximately 2.7 million lives as of March 15, 2021. Despite public health concerns presented by coronaviruses, progress in the development of therapeutics and vaccines for coronaviruses has been slow until recently.

To prevent the spread of COVID-19, safe and effective vaccines that induce potent and long-lasting virus-specific immune responses are needed [8–12]. Betacoronaviruses (Beta-CoVs), such as SARS-CoV-2, are enveloped, positive-sense, ssRNA viruses [13, 14]. BetaCoVs encode the envelope, nucleocapsid, membrane, and spike (S) proteins [15, 16]. Among these components, the spike protein has received considerable attention due to its proven role in the virus infection process [17]. The S protein of the viral envelope comprises two subunits, S1 and S2, that function in viral attachment to the host cell receptor and in fusion to the cells, respectively [17, 18]. For instance, the S protein on the envelope of SARS-CoV-2 binds to the cell receptor angiotensin converting enzyme 2 (ACE2) and facilitates viral entry [18, 19]. Importantly, it has been demonstrated with the two preceding Beta-CoVs (SARS-CoV and MERS-CoV) that antibodies targeting the S protein can block the binding of these viruses to the cell receptor, rendering the S protein an attractive target for vaccine development to elicit virus-specific neutralizing antibody responses, and in turn, protective immunity against coronaviruses [20]. Indeed, vaccine candidates based on the viral S protein have been previously developed for SARS-CoV and MERS-CoV, establishing the immunogenicity of the S protein of Beta-CoVs [21–23]. Furthermore, our previous efforts on the development of vaccines against MERS-CoV and SARS-CoV have shown that vaccine candidates targeting the S1 subunit are capable of generating efficacious neutralizing antibody responses [24, 25]. More recently, we have also presented that skin-targeted S1 subunit protein vaccines induce antigen-specific antibody responses against MERS-CoV and SARS-CoV-2 [8]. As such, the literature suggests that the S1 subunit is an important target for vaccine candidates against coronaviruses.

Remarkable progress in the fields of molecular biology and biotechnology has enabled production of novel vaccines to combat infectious diseases [26–28]. Recombinant DNA technology has proven a viable approach due to its versatility, cost-effectiveness, and logistic advantages [29–32]. Genetic immunization based on recombinant DNA or mRNA vaccines, where the intracellular delivery of nucleic acids enables the synthesis of a rationally selected antigen of pathogens to elicit virus-specific protective immunity, has been investigated toward combatting infectious diseases [11, 26, 33–38].

Engineered viral vectors have been an attractive alternative to nonviral transgene expression [39–41]. Adenovirus (Ad)-vectored vaccines encoding a target antigen gene have been increasingly used for their demonstrated capacity to induce both humoral and cellular immune responses [42–45]. The promising immunogenicity of Ad vaccines has been shown in several animal models and there are a number of Ad-based vaccine candidates against a myriad of pathogens currently being evaluated in clinical trials [46–49]. Interestingly, our previous studies have demonstrated that Ad-vectored vaccines expressing SARS-CoV-S1 and MERS-CoV-S1 antigens generate potent and efficacious antibody responses, making recombinant Ad-based vaccines an appealing candidate against emerging coronaviruses diseases [24, 25]. Importantly, several Ad-based SARS-CoV-2 vaccine candidates have recently emerged with promising results in large clinical trials [50–53], prompting the approval of a number of Ad-based SARS-CoV-2 vaccines for emergency use. In addition to the prevailing Ad serotype (Ad5), Ad26 and a chimpanzee adenovirus have been used for development of SARS-CoV-2 vaccines to overcome pre-existing Ad immunity [50–53]. Interestingly, recent clinical studies have demonstrated the promising immunogenicity of intramuscularly delivered Ad5-vectored COVID-19 vaccines encoding the gene for the full S protein [50, 51]. Despite the unprecedented progress with Ad-based SARS-CoV-2 vaccines, there is still an important need for development of alternative Ad-based vaccine candidates for COVID-19 and other infectious diseases, as well as for investigation of the different administration routes with new adenoviral vaccine candidates to enable sustainable global immunization programs.

Here, we describe the development of an Ad-vectored SARS-CoV-2 vaccine candidate for COVID-19 immunization. Based on our experience with Ad-based vaccines for prior BetaCoVs (SARS-CoV and MERS-CoV) [24, 25], we designed and constructed a recombinant type 5 Ad vector encoding the gene for the SARS-CoV-2-S1 subunit antigen (Ad5.SARS-CoV-2-S1). We evaluated the immunogenicity of the developed Ad vaccine in BALB/c mice through S.C. injection or I.N. delivery to test its ability to

induce antigen-specific humoral and cellular immune responses, and investigated virus-specific neutralization activity of the generated antibodies. Our study demonstrates the rational design and development of an Ad-based SARS-CoV-2 vaccine that is capable of eliciting robust and durable SARS-CoV-2-specific immune responses in mice, supporting the further development of recombinant adenovirus vaccines against COVID-19 and other emerging infectious diseases.

## Results

### Adenoviral SARS-CoV-2-S1 vaccine

To produce E1/E3 deleted, replication-deficient, human type 5 adenovirus expressing SARS-CoV-2-S1 protein, we generated pAd/SARS-CoV-2-S1 by subcloning the codon-optimized SARS-CoV-2-S1 gene into the shuttle vector, pAd (GenBank U62024) at SalI & NotI sites. Next, Ad5.SARS-CoV-2-S1 (Ad5.S1) was created by loxP homologous recombination (Fig. 1A). To detect SARS-CoV-2-S1 expression driven by the generated Ad candidate, the serum-free supernatants from A549 cells infected with Ad5.S1 were characterized by SDS-PAGE and Western blot analysis. The recombinant SARS-CoV-2-S1 proteins (both positive control and Ad5.S1-infected cells) were recognized by the polyclonal antibody at the expected glycosylated monomer molecular weights of about 110 kDa, while no expression was detected in the mock-infected cells (Fig. 1B).

### SARS-CoV-2-S1-specific antibody endpoint titers

To evaluate the immunogenicity of the constructed Ad5.SARS-CoV-2-S1 vaccine, we first determined antigen-specific IgG, IgG1, and IgG2a antibody endpoint titers in the sera of vaccinated mice (either via I.N. delivery or S.C. injection) and control mice (PBS or Adψ5 immunized groups). Serum samples, collected from all mice before immunization (Week 0) and subsequent weeks after vaccination, were serially diluted to determine SARS-CoV-2-S1-specific IgG, IgG1, and IgG2a endpoint titers for each immunization group using ELISA (Fig. 2). Results suggest that both I.N. and S.C. immunization induced significantly increased S1-specific IgG, IgG1, and IgG2a endpoint titers as early as 2 weeks after a single vaccination with Ad5.SARS-CoV-2-S1 compared to unimmunized groups (Fig. 2A-C, $p < 0.05$, Kruskal–Wallis test, followed by Dunn's multiple comparisons) and the elicited IgG, IgG1, and IgG2a antibody responses remained significantly higher with respect to control groups through week 24 (maximum length of the study to date) (Fig. 2 and Supporting information Fig. S1 present the results of two independent experiments to show the reproducibility of antibody responses elicited by I.N. and S.C. vaccination with Ad5.SARS-CoV-2-S1). Together, these results indicate that a single immunization with Ad5.SARS-CoV-2-S1 via either S.C. delivery or I.N. administration is capable of generating robust and long-lived S1-specific antibody responses, and the choice of the route of vaccine administration (S.C. or I.N.) had no significant effect on the generated antibody endpoint titers.

### Antigen-specific B-cell responses

To support the generation of long-lasting antibody responses against SARS-CoV-2 with our Ad-based vaccine, we performed studies to investigate vaccination-induced GC reactions. GC reactions are highly associated with generation of long-lived, high affinity antibody forming cells (AFC) and, hence, long-term humoral immunity. Mice were immunized with Ad5.SARS-CoV-2-S1, and S1-specific GC reactions and antibody-secreting plasma cells in the BM were measured through flow cytometry and ELISpot, respectively. I.N. and S.C. vaccination resulted in significant S1-specific GC reactions in cervical and axillary LNs of immunized mice, respectively, (Fig. 3A-D). As expected, neither S.C. vaccination with Ad5.SARS-CoV-2-S1, nor any of the control immunization groups induced GC reactions in cervical LNs (Fig. 3A), whereas I.N. vaccination with Ad5.SARS-CoV-2-S1 produced S1-specific B cells with GC phenotype in cervical LNs in eight out of ten mice, with Ig isotype switch to IgG1 and IgG2a (Fig. 3A and B). Nine out of ten mice vaccinated by S.C. injection of Ad.SARS-CoV-2-S1 displayed S1-specific GC B cells in axillary LNs, with induced class switch to IgG1 and IgG2a (Fig. 3C and D), whereas I.N. immunization with Ad.SARS-CoV-2-S1 did not result

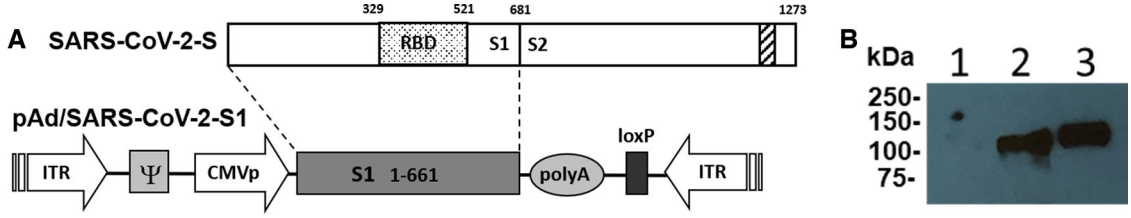


**Figure 1.** Adenoviral-vectored SARS-CoV-2-S1 vaccine. (A) A shuttle vector carrying the codon-optimized SARS-CoV-2-S1 gene encoding N-terminal 1–661 was designed as shown in the diagram. The vector was used to generate recombinant type 5 replication-deficient adenoviruses (Ad5) by homologous recombination with the adenoviral genomic DNA. ITR, inverted terminal repeat; RBD, receptor binding domain. (B) Detection of the SARS-CoV-2-S1 protein by western blot with the supernatant of A549 cells infected with Ad5.SARS-CoV-2.S1 (Ad5.S1) (10 MOI) using antispike protein of SARS-CoV rabbit polyclonal antibody (lane 2). Mock (AdΨ5)-infected cells were treated the same and used as a negative control (lane 1). As a positive control, 100 ng of recombinant SARS-CoV-2-S1 (Sino biological, 1–685 amino acids with ten histidine tag) was loaded (lane 3). The supernatants were resolved on SDS-10% polyacrylamide gel after being boiled in 2% SDS sample buffer with β-ME. The images of original Western blots used for preparation of Fig. 1B are shown in Supporting information Fig. S5.

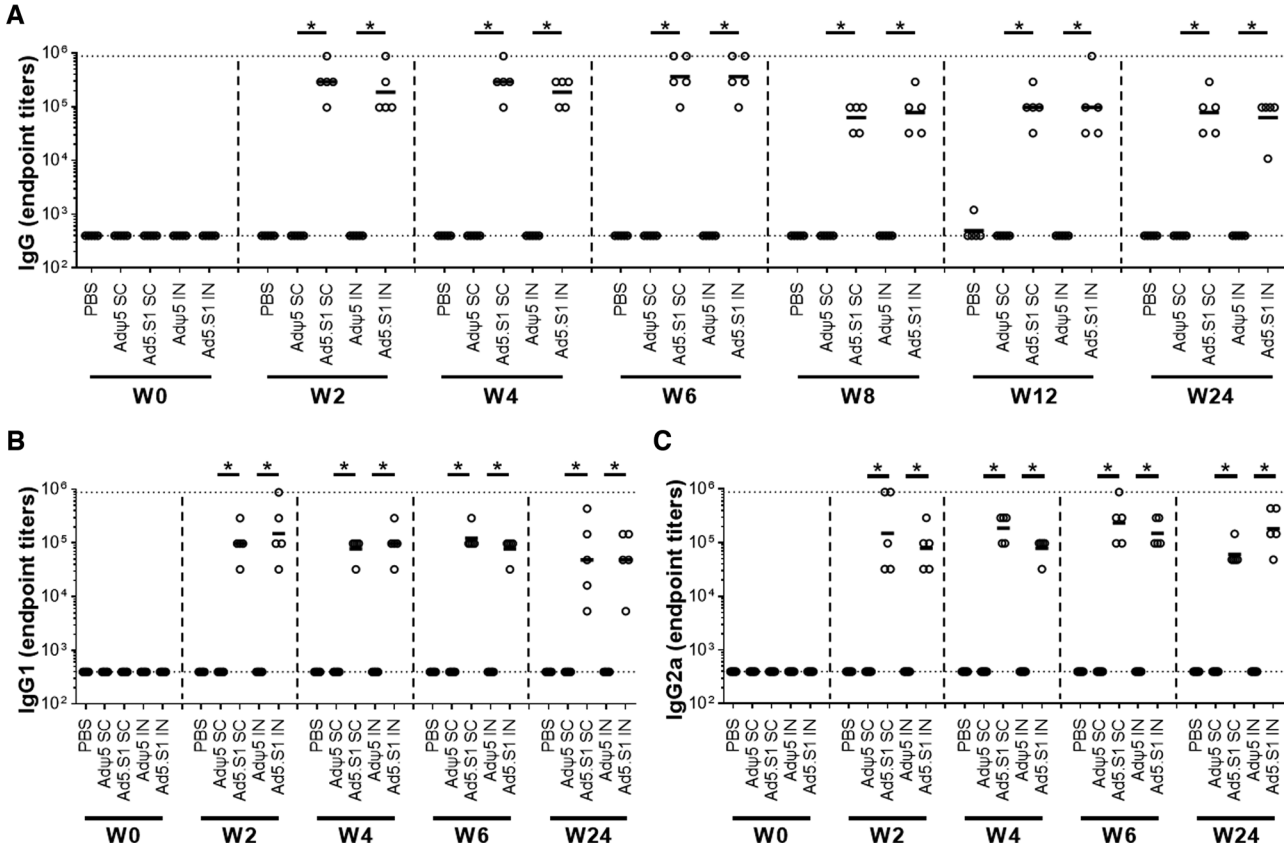


**Figure 2.** Antigen-specific antibody responses in mice immunized with adenoviral vectored SARS-CoV-2-S1 vaccine. BALB/c mice were immunized S.C. or I.N. with $1.5 \times 10^{10}$ vp of Ad5.SARS-CoV-2-S1 (Ad5.S1) or AdΨ5, while mice were immunized subcutaneously with PBS as a negative control. On weeks 0, 2, 4, 6, 8, 12, and 24 after vaccination, the sera from mice were collected, diluted, and SARS-CoV-2-S1-specific antibodies were quantified by ELISA to determine the (A) IgG (weeks 0, 2, 4, 6, 8, 12, and 24), (B) IgG1 (weeks 0, 2, 4, 6, and 24), and (C) IgG2a (weeks 0, 2, 4, 6, and 24) endpoint titers. Horizontal lines indicate geometric mean antibody titers. Significance was determined by Kruskal–Wallis test, followed by Dunn's multiple comparisons (*$p < 0.05$). Representative data are from one of two independent experiments (n = 5 mice per group for each experiment).

in GC reactions in axillary LNs (Fig. 3C). Preliminary results from ELISpot analysis showed that both I.N. and S.C. vaccination produced S1-specific antibody-producing plasma cells in the BM of immunized mice (Supporting information Fig. S3). Collectively, these results show that S.C. and I.N. immunization with Ad5-SARS-CoV-2-S1 are capable of forming significant antigen-specific GC reactions in draining LNs, which yielded detectable antigen-specific plasma cells in the BM of immunized mice, thereby suggesting that our Ad5.SARS-CoV-2-S1 vaccine has the potential to generate durable humoral immune effector cells, such as long-lived plasma cells.

## Antigen-specific cellular immune responses

To evaluate antigen-specific cellular immune responses induced by a single immunization of BALB/c mice with Ad5.SARS-CoV-2-S1, we investigated S1-specific cellular immunity in mice after vaccination by quantifying antigen-specific IFN-$\gamma^+$ $CD8^+$ and $CD4^+$ T-cell responses through intracellular cytokine staining (ICS) and flow cytometry. Results suggest that both I.N. and S.C. immunization elicited significantly enhanced systemic S1-specific $CD8^+$ and $CD4^+$ T-cell immunity compared to control groups. (Fig. 4, $p < 0.05$, one-way Welch's ANOVA, followed by Dunnett's T3 multiple comparisons). Interestingly, S.C. vaccination induced significantly increased systemic S1-specific IFN-$\gamma^+$ $CD8^+$ T-cell responses compared to I.N. immunization. (Fig. 4, $p < 0.05$, one-way Welch's ANOVA, followed by Dunnett's T3 multiple comparisons). Taken together, these findings indicate that a single vaccination with Ad5.SARS-CoV-2-S1 via either S.C. delivery or I.N. administration is capable of generating robust systemic S1-specific cellular immune responses, and the choice of the route of vaccine administration (S.C. or I.N.) has a significant effect on the Ad5.SARS-CoV-2-S1 vaccine-induced $CD8^+$ T-cell responses.

## SARS-CoV-2 neutralizing antibody titers

To evaluate the functional quality of vaccine-generated antigen-specific antibodies, we used a microneutralization assay ($NT_{90}$) to test the ability of sera from immunized mice to neutralize the infectivity of SARS-CoV-2. Sera, collected from all mice 8 and 12 weeks after vaccination, were tested for the presence of SARS-CoV-2-specific neutralizing antibodies, and the results are shown

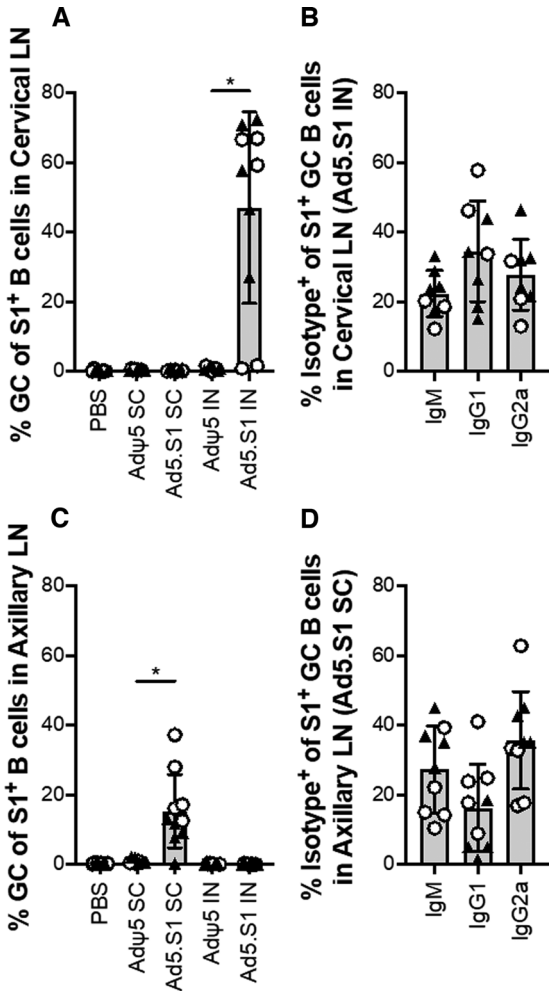


**Figure 3.** Antigen-specific humoral responses in mice immunized with Ad5.SARS-CoV-2-S1. Formation of GC reactions and Ig isotype switching in draining LNs. BALB/c mice were vaccinated S.C. or I.N. with $1.5 \times 10^{10}$ vp of Ad5.SARS-CoV-2-S1 (Ad5.S1) or AdΨ5, while mice were immunized S.C. with PBS as a negative control. Cervical and axillary LNs were harvested 14 days after I.N. and S.C. vaccination and single-cell suspensions of LNs were stained and analyzed by flow cytometry to determine the frequencies of S1-specific GC-B cells and their IgM, IgG1, and IgG2a isotype distribution. (A) S1-specific GC B cells in cervical LNs. (B) IgM, IgG1, and IgG2a isotype distribution of S1-specific B cells in cervical LNs. (C) S1-specific GC B cells in axillary LNs. (D) IgM, IgG1, and IgG2a isotype distribution of S1-specific B cells in axillary LNs. As outlined in the gating strategy presented in Supporting information Fig. S2, %GC of $S1^+$ B cells in **A** and **C** were calculated as %$CD95^+$ $CD38^-$ of live $CD19^+$ $S1^+$, and frequencies of isotype-specific GC B-cell subsets in **B** and **D** were calculated as %$Isotype^+$ of live $CD19^+$ $S1^+$ $CD95^+$ $CD83^-$ cells. Results are mean ± SD. Groups were compared by one-way Welch's ANOVA, followed by Dunnett's T3 multiple comparisons, and significant differences are indicated by $^*p < 0.05$. Data are from two independent experiments (n = 5 mice per group for each experiment) that are indicated by circles or triangles.

in Fig. 5. As expected, there were no detected neutralizing antibody responses in the sera of mice immunized with PBS or Adψ5 control groups, while SARS-CoV-2-neutralizating antibodies were detected in mice immunized by either I.N. delivery or S.C. injection of Ad5.SARS-CoV-2-S1 both 8 and 12 weeks after vaccination. The resulting SARS-CoV-2-neutralizing activity on weeks 8

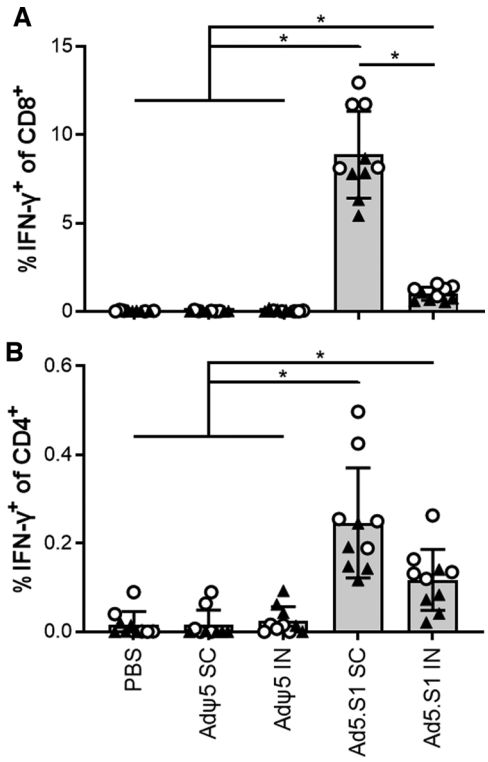


**Figure 4.** Antigen-specific cellular responses in mice immunized with Ad5.SARS-CoV-2-S1. BALB/c mice were immunized S.C. or I.N. with $1.5 \times 10^{10}$ vp of Ad5.SARS-CoV-2-S1 (Ad5.S1) or AdΨ5, or subcutaneously with PBS as a negative control. Twelve days after vaccination, splenocytes were isolated and stimulated with SARS-CoV-2 S1 PepTivator, followed by intracellular cytokine staining (ICS) and flow cytometry to identify SARS-CoV-2 S1-specific T cells (see Supporting information Fig. S4 for complete gating strategy). Frequencies of SARS-CoV-2 S1-specific (A) $CD8^+$ IFN-$\gamma^+$ and (B) $CD4^+$ IFN-$\gamma^+$ T cells, presented after subtracting background responses detected in corresponding unstimulated splenocyte samples. Results are mean ± SD. Groups were compared by one-way Welch's ANOVA, followed by Dunnett's T3 multiple comparisons, and significant differences are indicated by $^*p < 0.05$. Data are from two independent experiments (n = 5 mice per group for each experiment) that are indicated by circles or triangles.

and 12 after I.N. and S.C. immunization was statistically significant (Fig. 5, $p < 0.05$, Kruskal–Wallis test, followed by Dunn's multiple comparisons) compared to PBS control, with no significant differences with respect to each other.

## Discussion

Sustainable immunization programs against SARS-CoV-2 and other novel coronaviruses require cost-effective, patient-friendly, rapidly scalable, and clinically feasible vaccines that are capable of inducing long-term immunity after a single immunization. To address this continuing demand, our study presents the development of an Ad-based COVID-19 vaccine (Ad5.SARS-CoV-2-S1) and its immunogenicity in mice. Current leading Ad-based SARS-CoV-2 vaccines, Oxford-AstraZeneca, Janssen, CanSinoBio, and Sputnik V COVID-19 vaccines, encode the gene for full-length SARS-CoV-2 spike protein and are administered intramuscularly,

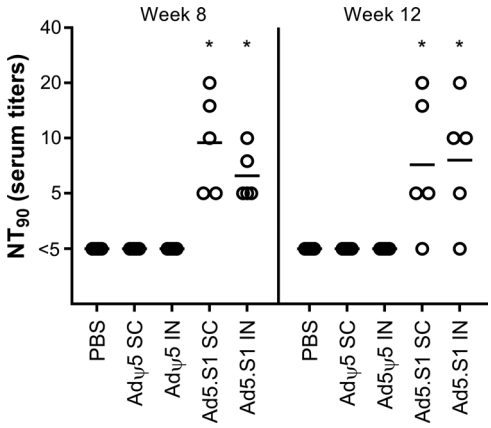


**Figure 5.** Neutralizing antibody responses in mice immunized with Ad5.SARS-CoV-2 S1. BALB/c mice were immunized S.C. or I.N. with $1.5 \times 10^{10}$ vp of Ad5.SARS-CoV-2-S1 (Ad5.S1) or AdΨ5, while mice were immunized subcutaneously with PBS as a negative control. Neutralizing antibodies in serum of mice 8 or 12 weeks after immunization were measured using a microneutralization assay ($NT_{90}$) with SARS-CoV-2. Serum titers that resulted in a 90% reduction in cytopathic effect compared to the virus control were reported. Horizontal lines represent geometric mean neutralizing antibody titers. Groups were compared by Kruskal–Wallis test at each time point, followed by Dunn's multiple comparisons. Significant differences relative to the PBS control are indicated by $^*p < 0.05$. The minimal titer tested was 5, and undetectable titers (those with $NT_{90}$ serum titers < 5) were assigned a value of 2.5. Data are from a single experiment (n = 5 mice per group).

whereas our vaccine encodes the gene for SARS-CoV-2-S1 subunit and is being tested for its immunogenicity via I.N and S.C administration. A single immunization of BALB/c mice via either I.N. or S.C. delivery of our Ad5.SARS-CoV-2-S1 vaccine elicited robust S1-specific humoral and cellular immune responses, where I.N. administration represents a minimally invasive option. Further improvements could be achieved with different immunization regimens including homologous or heterologous prime-boost vaccination strategies [54–56]. For instance, intracutaneous vaccination with microneedle arrays, which have been shown to deliver a broad range of recombinant DNA or protein vaccines, with or without adjuvants, could be utilized to achieve different prime-boost immunization strategies [57–60].

In support of long-lasting S1-specific antibody responses, our mechanistic studies suggest that a single vaccination of BALB/c mice via either I.N. administration or S.C. delivery of Ad5.SARS-CoV-2-S1 was capable of forming antigen-specific GC reactions and inducing Ig isotype switches to IgG1 and IgG2a in GCs in the corresponding draining LNs 14 days after vaccination. Further, these Ig-isotype switched GC reactions in draining LNs likely enabled the generation of S1-specific antibody-secreting plasma cell responses in the BM of immunized mice 6 weeks after immunization, as GCs are typically the source of long-lived humoral immune effector cells ensuring sustained antibody production [61–63]. These results are promising and support the use of Ad5.SARS-CoV-2-S1 vaccine against COVID-19 to induce antigen-specific GC reactions, leading to the generation of long-lived plasma cells and mutated memory B cells in response to a single vaccination.

We also analyzed S1-specific T-cell responses in the spleen of mice to investigate the capacity of Ad5.SARS-CoV-2-S1 administered by either S.C. or I.N. routes to elicit antigen-specific systemic cellular immune responses. A single vaccination via either I.N. or S.C. delivery of Ad5.SARS-CoV-2-S1 was capable of generating significant systemic cellular immune responses compared to unimmunized control groups. In agreement with a previous report [64], vaccination via S.C. route was more efficient in inducing systemic S1-specific $CD8^+$ T-cell responses compared to I.N. delivery. Our future work will include more comprehensive investigation of these differences to establish the impact of the route of Ad5.SARS-CoV-2-S1 administration on antigen-specific cellular immune responses. In addition, we will study the magnitude, kinetics, and types of S1-specific cellular immune responses in different organs, such as lungs and various draining LNs, to provide additional insight into the quality, breadth, and durability of protective T-cell responses induced by Ad5.SARS-CoV-2-S1 vaccine.

Neutralization assays are pivotal for testing the quality of the immunization-induced antibodies in terms of their ability to reduce the amount of infectious virus titer in culture. Here, we used a microneutralization test ($NT_{90}$) to evaluate the function of the generated antibodies in the sera of immunized mice and showed that a single immunization using either I.N. delivery or S.C. injection of Ad5.SARS-CoV-S1 vaccine was capable of inducing significant SARS-CoV-2-neutralizing antibody titers at weeks 8 and 12 after vaccination with respect to control groups. If needed, it may be possible to further improve neutralizing antibody responses with different prime-boost vaccination strategies. Further, clinical translation of Ad vaccines has been predominantly hampered by pre-existing immunity against the viral capsid, which diminishes vaccine efficacy [65]. Notably, a recent study demonstrated that intramuscular immunization with a recombinant type 5 adenovirus vaccine encoding the gene for the full spike protein could overcome the pre-existing vector immunity [50]. Thus, immunization with Ad-based vaccines could be a feasible alternative to combat emerging infectious respiratory diseases including COVID-19.

BALB/c mice have been widely used to investigate the immunogenicity of different vaccines against coronaviruses [8, 24, 36, 66], thereby representing a reliable model for the immunogenicity testing of Ad5.SARS-CoV-2-S1. The rational design and construction of our Ad5.SARS-CoV-2-S1 vaccine resulted in promising immune responses in BALB/c mice; however, it will still be important to test the immunogenicity of Ad5.SARS-CoV-2-S1 in different mouse strains and especially in larger animal models to extrapolate these responses to human studies. Our future studies will include animal challenge models with more detailed T- and B-cell studies. Two recent studies have investigated viral replication and clearance after challenge in rhesus macaque and guinea pig models and demonstrated promising results [36, 67]. We are currently working on the development and validation of a transgenic hACE2 mouse model to perform protection studies in the future.

In sum, our Ad-based vaccine induces significant antigen-specific humoral and cellular immune responses against

SARS-CoV-2. These results suggest that Ad-based vaccines have the potential to be versatile candidates for the induction of virus-specific protective immune responses against COVID-19 and other emerging infectious diseases.

## Materials and methods

### Construction of recombinant adenoviral vectors

The coding sequence for SARS-CoV-2-S1 amino acids 1 to 661 of full length from BetaCoV/Wuhan/IPBCAMS-WH-05/2020 (GISAID accession id. EPI_ISL_403928) flanked with SalI & NotI was codon-optimized using the UpGene algorithm for optimal expression in mammalian cells [68] and synthesized (GenScript). pAd/SARS-CoV-2-S1 was then created by subcloning the codon-optimized SARS-CoV-2-S1 gene into the shuttle vector, pAdlox (GenBank U62024), at SalI/NotI sites. Subsequently, replication-deficient human recombinant serotype 5 adenovirus vector (Ad5.SARS-CoV-2-S1) was generated by loxP homologous recombination and purified [24, 69, 70].

### SDS-PAGE and western blot

To evaluate the infectivity of the constructed recombinant adenoviruses, A549 (human lung adenocarcinoma epithelial cell line) cells were transduced with a MOI of 10 of Ad5.SARS-CoV-2-S1. At 6 h after infection, cells were washed three times with PBS, and then incubated with serum-free media for 48 h. The supernatants of A549 cells transduced with Ad5.SARS-CoV-2-S1 were subjected to SDS-PAGE and Western blot. Briefly, after the supernatants were boiled in Laemmli sample buffer containing 2% SDS with beta-mercaptoethanol (β-ME), the proteins were separated by Tris-Glycine SDS-PAGE gels and transferred to nitrocellulose membrane. After blocking for 1 h at room temperature (RT) with 5% nonfat milk in PBS-T, rabbit anti-SARS-CoV spike polyclonal antibody (1:3000) (Sino Biological) was added and incubated overnight at 4°C as primary antibody, and HRP-conjugated goat anti-rabbit IgG (1:10 000) (Jackson immunoresearch) was added and incubated at RT for 2 h as secondary antibody. After washing three times with PBS, the signals were visualized using ECL Western blot substrate reagents and Amersham Hyperfilm (GE Healthcare). Mock (AdΨ5)-infected A549 cells and 100 ng of recombinant SARS-CoV-2-S1 (Sino biological, 1–685 amino acids with ten histidine tag) were used as negative and positive controls, respectively.

### Animals and immunization

BALB/cJ mice (n = 5 animals per group in each independent experiment unless otherwise noted) were vaccinated by either S.C. injection or I.N. delivery of $1.5 \times 10^{10}$ viral particles (vp) of AdΨ5 (a null Ad5 vector control) or, Ad5.SARS-CoV-2-S1, or by S.C. injection of PBS as a negative control. Mice were bled from retro-orbital vein at weeks 0, 2, 4, 6, 8, 12, and 24 after immunization, and the obtained serum samples were diluted and used to evaluate S1-specific antibodies by ELISA. Serum samples obtained on weeks 8 and 12 after vaccination were also used for microneutralization (NT) assay. Mice were maintained under specific pathogen-free conditions at the University of Pittsburgh, and all experiments were conducted in accordance with animal use guidelines and protocols approved by the University of Pittsburgh's Institutional Animal Care and Use (IACUC) Committee.

### ELISA

Sera from all mice were collected prior to immunization (week 0) and every two weeks (weeks 2, 4, 6) after immunization and evaluated for SARS-CoV-2-S1-specific IgG, IgG1, and IgG2a antibodies using ELISA [8]. Further, sera from all mice collected at weeks 8, 12, and 24 after immunization were tested for SARS-CoV-2-S1-specific IgG antibodies using ELISA for long-term humoral responses. Sera collected at week 24 after vaccination were also tested for SARS-CoV-2-S1-specific IgG1 and IgG2a antibodies using ELISA. Briefly, ELISA plates were coated with 200 ng of recombinant SARS-CoV-2-S1 protein (Sino Biological) per well overnight at 4°C in carbonate coating buffer (pH 9.5) and then blocked with PBS-T and 2% BSA for 1 h. Mouse sera were diluted in PBS-T with 1% BSA and incubated for 72 h. After the plates were washed, anti-mouse IgG-HRP (1:2000, SantaCruz) or anti-mouse IgM-HRP (1:5000, Jackson Immunoresearch) were added to each well and incubated for 1 h. The plates were washed three times, developed with 3,3'5,5'-tetramethylbenzidine, and the reaction was stopped. Next, absorbance was determined at 450 nm using a plate reader. For IgG1 and IgG2a ELISAs, mouse sera were diluted in PBS-T with 1% BSA and incubated for 72 h. After the plates were washed, biotin-conjugated IgG1 and IgG2a (1:1000, eBioscience) and streptavidin alkaline phosphatase (1:500, PharMingen) were added to each well and incubated for 1 h. The plates were washed three times and developed with para-nitrophenylphosphate, and the reaction was stopped and absorbance at 405 nm was determined using a plate reader.

### Flow cytometry analysis for humoral immune responses

In order to address the humoral immune response, mice were sacrificed 14 days after vaccination and single cell suspensions of draining LNs were analyzed by flow cytometry, adhering to the recently published guidelines [71]. We biotinylated SARS-CoV-2-S1 protein, which allowed us to use it as "bait" in flow cytometric analysis to identify antigen-specific B cells, formation of GC reactions, and immunoglobulin isotype switching in

draining LNs. Lymph nodes were disrupted by crushing them between frosted glass slides in staining buffer (SB; PBS/2%FCS/2 mM EDTA). Single cell suspensions were subjected to viability staining using Zombie NIR fixable viability dye [FVD] (BioLegend) for 15 min on ice and then incubated with anti-CD16/CD32 Abs in SB for 5 min on ice to block Fc receptors. Cells were then stained in SB with antibodies against CD19-BV786 (clone 1D3), CD38-Al594 (clone 90), CD95-PE-Cy7 (clone Jo2), IgM-Al680 (clone B7-6), IgG1-V450 (clone A-85), IgG2a-Al488 (goat polyclonal; Southern Biotech), and biotinylated-SARS-CoV-2-S1. Cells were washed and stained with Streptavidin PE (PROzyme) for 15 min and then washed and fixed with 1% PFA over night at 4°C before data acquisition (0.5-1 $\times$ $10^6$ cells per flow stain) on the Cytek™ Aurora Cytometer (Cytek Biosciences). For flow cytometric analysis, SARS-CoV-2-S1-specific GC B cells were defined as live singlets (fixable viability dye$^{neg}$) and consecutively gated as SARS-CoV-2-S1$^{pos}$, CD19$^{pos}$, CD38$^{neg}$, CD95$^{pos}$ using the software FlowJo Version 10. The corresponding gating strategy is shown in Supporting information Fig. S2.

## ELISpot for antibody-secreting cells

The frequency of SARS-CoV-2-specific antibody producing cells in the BM of mice was determined by ELISpot assay 6 weeks after immunization using our established and previously published methods [63, 72]. Briefly, 4-HBX plates were coated as described for ELISA assays, and nonspecific binding was blocked with RPMI media containing 5% FCS. Cells were plated at the indicated density and incubated at 37°C for 5 h. Secondary Ab (anti-mIgG-alkaline phosphatase; Southern Biotech) was detected using 5-bromo-4-chloro-3-indolyl phosphate substrate (BCIP; Southern Biotech) in 0.5% low melting agarose (Fisher Scientific). Spots were counted using a binocular on a dissecting microscope and the detected numbers of IgG anti-SARS-CoV-2-S1 AFCs were calculated per million BM cells. The picture of the ELISpot plate was prepared in Photoshop.

## Flow cytometry analysis for cellular immune responses

Antigen-specific T-cell responses in the spleen of BALB/c mice immunized as described above were analyzed 12 days after immunization by flow cytometry, adhering to the recently published guidelines [71]. A number of previous studies also investigated systemic cellular immune responses induced by Ad-based vaccines in the spleen of mice at day 12 postimmunization [45, 73, 74]. Splenocytes isolated from vaccinated and PBS control mice were stimulated with PepTivator SARS-CoV-2-S1 (a pool of S1 MHC class I- and MHC class II-restricted peptides) for 6 h in the presence of protein transport inhibitors (Brefeldin A + Monensin) for the last 4 h. Unstimulated cells were used as negative controls. Cells were stained with antibodies for CD4 (GK1.5, BUV395, BD Biosciences), CD8b (H35-17.2, BUV737, BD Biosciences), and a (FVD, eFluor 780, eBioscience), followed by ICS using a Fix & Perm Cell Permeabilization Kit (Invitrogen) and IFN-γ antibody (XMG1.2, BV421, BD Biosciences). Data were collected and analyzed using a BD LSR II cytometer and FlowJo v10 software (BD Biosciences). Live, antigen-specific, IFN-γ-producing CD8$^+$ and CD4$^+$ T cells were identified according to the gating strategy in Supporting information Fig. S4. Frequencies of IFN-γ$^+$ cells from unstimulated controls were subtracted from corresponding peptide-stimulated samples, and any negative values set to zero.

## SARS-CoV-2 microneutralization assay

Neutralizing antibody (NT-Ab) titers against SARS-CoV2 were defined according to the following protocol [75, 76]. Briefly, 50 μL of sample from each mouse, in different dilutions, were added in two wells of a flat bottom tissue culture microtiter plate (COSTAR, Corning Incorporated, NY 14831, USA), mixed with an equal volume of 50 TCID50 of a SARS-CoV2 chinese strain isolated from a symptomatic chinese patient, previously titrated and incubated at 33°C in 5% $CO_2$. All dilutions were made in Eagle's Minimum Essential Medium with addition of 1% penicillin, streptomycin, and glutamine and 5 γ/mL of trypsin. After 1 h incubation at 33°C 5% $CO_2$, 3 $\times$ $10^4$ VERO E6 cells [VERO C1008 (Vero 76, clone E6, Vero E6); ATCC® CRL-1586™] were added to each well. After 72 hours of incubation at 33°C 5% CO2 wells were stained with Gram's crystal violet solution (Merck KGaA, 64271 Damstadt, Germany) plus 5% formaldehyde 40% m/v (Carlo ErbaSpA, Arese (MI), Italy) for 30 min. Microtiter plates were then washed in running water. Wells were scored to evaluate the degree of cytopathic effect (CPE) compared to the virus control. Blue staining of wells indicated the presence of neutralizing antibodies. Neutralizing titer was the maximum dilution with the reduction of 90% of CPE. A positive titer was equal or greater than 1:5. Sera from mice before vaccine administration were always included in microneutralizaiton (NT) assay as a negative control.

## Statistical analysis

Statistical analyses were performed using GraphPad Prism v9 (San Diego, CA). Antibody endpoint titers and neutralization data were analyzed by Kruskal–Wallis test, followed by Dunn's multiple comparisons. B- and T-cell data were analyzed by one-way Welch's ANOVA, followed by Dunnett's T3 multiple comparisons. Significant differences are indicated by $*p < 0.05$. Comparisons with nonsignificant differences are not indicated.

**Acknowledgments:** AG is funded by NIH Grants (UM1-AI106701, R21-AI130180, U01-CA233085) and UPMC Enterprises IPA 25565. LDF is funded by NIH Grants (UM1-AI106701,

R01-AR074285, R01-AR071277) and UPMC Enterprises IPA 25565. MJS is funded by NIH Grants (R01-AI137132 and R01-AI43603). AG, LDF, and MJS acknowledge the support from the University of Pittsburgh Clinical and Translational Science Institute (CTSI) and the DSF Charitable Foundation. These funding institutions had no role in the study design, data collection, data analysis, and interpretation of this publication. The authors acknowledge BioRender for the preparation of the Graphical Abstract.

**Author contribution:** Conceptualization: EK, MJS, LDF, AG; Data Curation: EK, FJW, SCB, SMJ, EP, IC, FB; Formal Analysis: EK, FJW, SCB, Investigation: EK, FJW, SCB, MSK, SH, GE, TWK, CDC, SMJ, LJC, NMW, NMAO, BLH, EP, IC, FB; Methodology: EK, FJW, MJS, LDF, AG; Resources: MJS, LDF, AG; Visualization: EK, FJW, SCB; Original Draft Preparation: EK, FJW, SCB; Review and Editing: MSK, SH, GE, TWK, CDC, SMJ, LJC, NMW, NMAO, BLH, EP, IC, FB, EK, MJS, LDF, AG.

**Conflict of interest:** The authors declare no commercial or financial conflict of interest.

**Peer review:** The peer review history for this article is available at https://publons.com/publon/10.1002/EJI.202149167.

**Data availability statement:** The data are available from the corresponding author upon reasonable request.

## References

1 **Raoult, D., Zumla, A., Locatelli, F., Ippolito, G. and Kroemer, G.**, Coronavirus infections: epidemiological, clinical, and immunological features and hypotheses. *Cell Stress*. 2020. **4**: 66–75.

2 **de Wit, E., van Doremalen, N., Falzarano, D. and Munster, V. J.**, SARS and MERS: recent insights into emerging coronaviruses. *Nat. Rev. Microbiol.* 2016. **14**: 523–534.

3 **Hilgenfeld, R. and Peiris, M.**, From SARS to MERS: 10 years of research on highly pathogenic human coronaviruses. *Antiviral Res.* 2013. **100**: 286–295.

4 **Acter, T., Uddin, N., Das, J., Akhter, A., Choudhury, T. R. and Kim, S.**, Evolution of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) as coronavirus disease 2019 (COVID-19) pandemic: a global health emergency. *Sci. Total Environ.* 2020. **730**: 138996.

5 **Lai, C. C., Shih, T. P., Ko, W. C., Tang, H. J. and Hsueh, P. R.**, Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and coronavirus disease-2019 (COVID-19): the epidemic and the challenges. *Int. J. Antimicrob. Agents*. 2020. **55**: 105924.

6 **Chakraborty, I. and Maity, P.**, COVID-19 outbreak: migration, effects on society, global environment and prevention. *Sci. Total Environ.* 2020. **728**: 138882.

7 **Bandyopadhyay, S.**, Coronavirus disease 2019 (COVID–19): we shall overcome. *Clean Technol. Environ. Policy*. 2020. **22**: 545–546.

8 **Kim, E., Erdos, G., Huang, S., Kenniston, T. W., Balmert, S. C., Carey, C. D., Raj, V. S. et al.**, Microneedle array delivered recombinant coronavirus vaccines: immunogenicity and rapid translational development. *EBioMedicine*. 2020. **55**: 102743.

9 **Lurie, N., Saville, M., Hatchett, R. and Halton, J.** Developing Covid-19 vaccines at pandemic speed. *N. Engl. J. Med.* 2020. **382**: 1969–1973.

10 **Chen, W. H., Strych, U., Hotez, P. J. and Bottazzi, M. E.**, The SARS-CoV-2 vaccine pipeline: an overview. *Curr. Trop. Med. Rep*. 2020. **7**: 61–64.

11 **Amanat, F. and Krammer, F.**, SARS-CoV-2 vaccines: status report. *Immunity*. 2020. **52**: 583–589.

12 **Lu, S.**, Timely development of vaccines against SARS-CoV-2. *Emerg Microbes Infect*. 2020. **9**: 542–544.

13 **Schoeman, D. and Fielding, B. C.**, Coronavirus envelope protein: current knowledge. *Virol. J*. 2019. **16**: 69.

14 **Khan, S., Siddique, R., Shereen, M. A., Ali, A., Liu, J., Bai, Q., Bashir, N. and Xue, M.**, Emergence of a novel coronavirus, Severe Acute Respiratory Syndrome Coronavirus 2: biology and therapeutic options. *J. Clin. Microbiol.* 2020. **58**: e00187-20.

15 **Tang, T., Bidon, M., Jaimes, J. A., Whittaker, G. R. and Daniel, S.**, Coronavirus membrane fusion mechanism offers a potential target for antiviral development. *Antiviral Res.* 2020. **178**: 104792.

16 **Li, X., Luk, H. K. H., Lau, S. K. P. and Woo, P. C. Y.**, Human coronaviruses: general features. *Ref. Mod. Biomed. Sci*. 2019. https://doi.org/10.1016/B978-0-12-801238-3.95704-0

17 **Walls, A. C., Park, Y. J., Tortorici, M. A., Wall, A., McGuire, A. T. and Veesler, D.**, Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein. *Cell*. 2020. **181**: 281–292.

18 **Yan, R., Zhang, Y., Li, Y., Xia, L., Guo, Y. and Zhou, Q.**, Structural basis for the recognition of SARS-CoV-2 by full-length human ACE2. *Science*. 2020. **367**: 1444–1448.

19 **Wang, Q., Zhang, Y., Wu, L., Niu, S., Song, C., Zhang, Z., Lu, G. et al.**, Structural and functional basis of SARS-CoV-2 entry by using human ACE2. *Cell*. 2020. **181**: 894–904.

20 **Jiang, S., Hillyer, C. and Du, L.**, Neutralizing antibodies against SARS-CoV-2 and other human coronaviruses. *Trends Immunol.* 2020. **41**: 355–359.

21 **Song, F., Fux, R., Provacia, L. B., Volz, A., Eickmann, M., Becker, S., Osterhaus, A. D. M. E. et al.**, Middle east respiratory syndrome coronavirus spike protein delivered by modified *vaccinia* virus ankara efficiently induces virus-neutralizing antibodies. *J. Virol.* 2013. **87**: 11950-11954.

22 **Coleman, C. M., Liu, Y. V., Mu, H., Taylor, J. K., Massare, M., Flyer, D. C., Glenn, G. M. et al.**, Purified coronavirus spike protein nanoparticles induce coronavirus neutralizing antibodies in mice. *Vaccine*. 2014. **32**: 3169–3174.

23 **Yong, C. Y., Ong, H. K., Yeap, S. K., Ho, K. L. and Tan, W. S.**, Recent advances in the vaccine development against middle east respiratory syndrome-coronavirus. *Front. Microbiol*. 2019. **10**: 1781.

24 **Kim, E., Okada, K., Kenniston, T., Raj, V. S., AlHajri, M. M., Farag, E. A. B. A., AlHajri, F. et al.**, Immunogenicity of an adenoviral-based middle east respiratory syndrome coronavirus vaccine in BALB/c mice. *Vaccine*. 2014. **32**: 5975–5982.

25 **Gao, W., Tamin, A., Soloff, A., D'Aiuto, L., Nwanegbo, E., Robbins, P. D., Bellini, W. J. et al.**, Effects of a SARS-associated coronavirus vaccine in monkey. *Lancet*. 2003. **362**: 1895–1896.

26 **Zhang, C., Maruggi, G., Shan, H. and Li, J.**, Advances in mRNA vaccines for infectious diseases. *Front. Immunol.* 2019. **10**: 594.

27 **Gary, E. N. and Weiner, D. B.**, DNA vaccines: prime time is now. *Curr. Opin. Immunol.* 2020. **65**: 21–27.

28 **Mascola, J. R. and Fauci, A. S.**, Novel vaccine technologies for the 21st century. *Nat. Rev. Immunol.* 2020. **20**: 87–88.

29 **Lee, J., Kumar, S. A., Jhan, Y. Y. and Bishop, C. J.**, Engineering DNA vaccines against infectious diseases. *Acta Biomater.* 2018. **80**: 31–47.

30 **Ivory, C. and Chadee, K.**, DNA vaccines: designing strategies against parasitic infections. *Genet. Vaccines Ther*. 2004. **2**: 17.

31 **Ghaffarifar, F.**, Plasmid DNA vaccines: where are we now? *Drugs Today*. 2018. **54**: 315–333.

32 **Condon, C., Watkins, S. C., Celluzzi, C. M., Thompson, K. and Falo Jr, L. D.**, DNA-based immunization by in vivo transfection of dendritic cells. *Nat. Med*. 1996. **2**: 1122–1128.

33 **Maslow, J. N.**, Vaccine development for emerging virulent infectious diseases. *Vaccine*. 2017. **35**: 5437–5443.

34 **Martin, J. E., Louder, M. K., Holman, L. A., Gordon, I. J., Enama, M. E., Larkin, B. D., Andrews, C. A. et al.**, A SARS DNA vaccine induces neutralizing antibody and cellular immune responses in healthy adults in a phase I clinical trial. *Vaccine*. 2008. **26**: 6338–6343.

35 **Liu, S., Wang, S. and Lu, S.**, DNA immunization as a technology platform for monoclonal antibody induction. *Emerg. Microbes Infect*. 2016. **5**: e33.

36 **Smith, T. R. F., Patel, A., Ramos, S., Elwood, D., Zhu, X., Yan, J., Gary, E. N. et al.**, Immunogenicity of a DNA vaccine candidate for COVID-19. *Nat. Commun.* 2020. **11**: 2601.

37 **Poland, G. A., Ovsyannikova, I. G., Crooke, S. N. and Kennedy, R. B.**, SARS-CoV-2 vaccine development: current status. *Mayo Clin. Proc.* 2020. **95**: 2172–2188.

38 **Pardi, N., Hogan, M. J. and Porter, F., Weissman, D.**mRNA vaccines—a new era in vaccinology. *Nat. Rev. Drug Discov.* 2018. **17**: 261–279.

39 **Ewer, K. J., Lambe, T., Rollier, C. S., Spencer, A. J., Hill, A. V. S. and Dorrell, L.**, Viral vectors as vaccine platforms: from immunogenicity to impact. *Curr. Opin. Immunol.* 2016. **41**: 47–54.

40 **Draper, S. J. and Heeney, J. L.**, Viruses as vaccine vectors for infectious diseases and cancer. *Nat. Rev. Microbiol.* 2010. **8**: 62–73.

41 **He, Y., Zhang, J., Donahue, C. and Falo Jr, L. D.**, Skin-derived dendritic cells induce potent CD8+ T cell immunity in recombinant lentivector-mediated genetic immunization. *Immunity*. 2006. **24**: 643–656.

42 **Gilbert, S. C. and Warimwe, G. M.**, Rapid development of vaccines against emerging pathogens: the replication-deficient simian adenovirus platform technology. *Vaccine*. 2017. **35**: 4461–4464.

43 **Van Kampen, K. R., Shi, Z., Gao, P., Zhang, J., Foster, K. W., Chen, D. T., Marks, D. et al.**, Safety and immunogenicity of adenovirus-vectored nasal and epicutaneous influenza vaccines in humans. *Vaccine*. 2005. **23**: 1029–1036.

44 **Stanley, D. A., Honko, A. N., Asiedu, C., Trefry, J. C., Lau-Kilby, A. W., Johnson, J. C., Hensley, L. et al.**, Chimpanzee adenovirus vaccine generates acute and durable protective immunity against ebolavirus challenge. *Nat. Med.* 2014. **20**: 1126–1129.

45 **Erdos, G., Balmert, S. C., Carey, C. D., Falo, G. D., Patel, N. A., Zhang, J., Gambotto, A. et al.**, Improved cutaneous genetic immunization by microneedle array delivery of an adjuvanted adenovirus vaccine *J. Invest. Dermatol.* 2020. **140**: 2528–2531.

46 **Ledgerwood, J. E., DeZure, A. D., Stanley, D. A., Coates, E. E., Novik, L., Enama, M. E., Berkowitz, N. M. et al.**, Chimpanzee adenovirus vector Ebola vaccine. *N. Engl. J. Med.* 2017. **376**: 928–938.

47 **De Santis, O., Audran, R., Pothin, E., Warpelin-Decrausaz, L., Vallotton, L., Wuerzner, G., Cochet, C. et al.**, Safety and immunogenicity of a chimpanzee adenovirus-vectored Ebola vaccine in healthy adults: a randomised, double-blind, placebo-controlled, dose-finding, phase 1/2a study. *Lancet Infect. Dis.* 2016. **16**: 311–320.

48 **Tatsis, N. and Ertl, H. C. J.**, Adenoviruses as vaccine vectors. *Mol. Ther.* 2004. **10**: 616–629.

49 **Lane, R.**, Sarah Gilbert: carving a path towards a COVID-19 vaccine. *Lancet*. 2020. **395**: 1247.

50 **Zhu, F. C., Li, Y. H., Guan, X. H., Hou, L. H., Wang, W. J., Li, J. X., Wu, S. P. et al.**, Safety, tolerability, and immunogenicity of a recombinant adenovirus type-5 vectored COVID-19 vaccine: a dose-escalation, open-label, non-randomised, first-in-human trial. *Lancet*. 2020. **395**: 1845–1854.

51 **Zhu, F. C., Guan, X. H., Li, Y. H., Huang, J. Y., Jiang, T., Hou, L. H., Li, J. X. et al.**, Immunogenicity and safety of a recombinant adenovirus type-5-vectored COVID-19 vaccine in healthy adults aged 18 years or older: a randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet*. 2020. **396**: 479–488.

52 **Ramasamy, M. N., Minassian, A. M., Ewer, K. J., Flaxman, A. L., Folegatti, P. M., Owens, D. R., Cathie, K. et al.**, Safety and immunogenicity of ChAdOx1 nCoV-19 vaccine administered in a prime-boost regimen in young and old adults (COV002): a single-blind, randomised, controlled, phase 2/3 trial. *Lancet*. 2020. **396**: 1979–1993.

53 **Sadoff, J., Le Gars, M., Shukarev, G., Heerwegh, D., Truyers, C., de Groot, A. M., Stoop, J. et al.**, Interim results of a phase 1–2a trial of Ad26.COV2.S Covid-19 vaccine. *N. Engl. J. Med.* 2020. https://doi.org/10.1056/NEJMoa2034201.

54 **Kardani, K., Bolhassani, A. and Shahbazi, S.**, Prime-boost vaccine strategy against viral infections: mechanisms and benefits. *Vaccine*. 2016. **34**: 413–423.

55 **Jung, S. Y., Kang, K. W., Lee, E. Y., Seo, D. W., Kim, H. L., Kim, H., Kwon, T. et al.**, Heterologous prime-boost vaccination with adenoviral vector and protein nanoparticles induces both Th1 and Th2 responses against Middle East respiratory syndrome coronavirus. *Vaccine*. 2018. **36**: 3468–3476.

56 **Lu, S.**, Heterologous prime-boost vaccination. *Curr. Opin. Immunol.* 2009. **21**: 346–351.

57 **Balmert, S. C., Carey, C. D., Falo, G. D., Sethi, S. K., Erdos, G., Korkmaz, E. and Falo Jr, L. D.**, Dissolving undercut microneedle arrays for multicomponent cutaneous vaccination. *J. Control. Release*. 2020. **317**: 336–346.

58 **Bachy, V., Hervouet, C., Becker, P. D., Chorro, L., Carlin, L. M., Herath, S., Papagatsias, T. et al.**, Langerin negative dendritic cells promote potent CD8+ T-cell priming by skin delivery of live adenovirus vaccine microneedle arrays. *Proc. Natl. Acad. Sci. U.S.A.* 2013. **110**: 3041–3046.

59 **Korkmaz, E., Balmert, S. C., Sumpter, T. L., Carey, C. D., Erdos, G. and Falo Jr, L. D.**, Microarray patches enable the development of skin-targeted vaccines against COVID-19. *Adv. Drug. Deliv. Rev.* 2021. **171**: 164–186.

60 **DeMuth, P. C., Li, A. V., Abbink, P., Liu, J., Li, H., Stanley, K. A., Smith, K. M. et al.**, Vaccine delivery with microneedle skin patches in nonhuman primates. *Nat. Biotechnol.* 2013. **31**: 1082–1085.

61 **Slifka, M. K. and Ahmed, R.**, Long-lived plasma cells: a mechanism for maintaining persistent antibody production. *Curr. Opin. Immunol.* 1998. **10**: 252–258.

62 **Shlomchik, M. J. and Weisel, F.**, Germinal center selection and the development of memory B and plasma cells. *Immunol. Rev.* 2012. **247**: 52–63.

63 **Weisel, F. J., Zuccarino-Catania, G. V., Chikina, M. and Shlomchik, M. J.**, A temporal switch in the germinal center determines differential output of memory B and plasma cells. *Immunity*. 2016. **44**: 116–130.

64 **Hoegh-Petersen, M., Thomsen, A. R., Christensen, J. P. and Holst, P. J.**, Mucosal immunization with recombinant adenoviral vectors expressing murine gammaherpesvirus-68 genes M2 and M3 can reduce latent viral load. *Vaccine*. 2009. **27**: 6723–6730.

65 **Thacker, E. E., Timares, L. and Matthews, Q. L.**, Strategies to overcome host immunity to adenovirus vectors in vaccine development. *Expert Rev. Vaccines*. 2009. **8**: 761–777.

66 **Bos, R., Rutten, L., van der Lubbe, J. E. M., Bakkers, M. J. G., Hardenberg, G., Wegmann, F., Zuijdgeest, D. et al.**, Ad26 vector-based COVID-19 vaccine encoding a prefusion-stabilized SARS-CoV-2 Spike immunogen induces potent humoral and cellular immune responses. *NPJ Vaccines*. 2020. **5**: 91.

67 **Yu, J., Tostanoski, L. H., Peter, L., Mercado, N. B., McMahan, K., Mahrokhian, S. H., Nkolola, J. P. et al.**, DNA vaccine protection against SARS-CoV-2 in rhesus macaques. *Science*. 2020. **369**: 806–811.

68 **Gao, W., Rzewski, A., Sun, H., Robbins, P. D. and Gambotto, A.**, UpGene: application of a web-based DNA codon optimization algorithm. *Biotechnol. Prog.* 2004. **20**: 443–448.

69 **Kim, E., Erdos, G., Huang, S., Kenniston, T., Falo Jr, L. D. and Gambotto, A.**, Preventative vaccines for zika virus outbreak: preliminary evaluation. *EBioMedicine*. 2016. **13**: 315–320.

70 **Hardy, S., Kitamura, M., Harris-Stansil, T., Dai, Y. and Phipps, M. L.**, Construction of adenovirus vectors through Cre-lox recombination. *J. Virol.* 1997. **71**: 1842-1849.

71 **Cossarizza, A., Chang, H. D., Radbruch, A., Acs, A., Adam, D., Adam-Klages, S., Agace, W. W. et al.**, Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). *Eur. J. Immunol.* 2019. **49**: 1457–1973.

72 **Good-Jacobson, K. L., Szumilas, C. G., Chen, L., Sharpe, A. H., Tomayko, M. M. and Shlomchik, M. J.**, PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat. Immunol.* 2010. **11**: 535–542.

73 **Rodrigues, E. G., Zavala, F., Eichinger, D., Wilson, J. M. and Tsuji, M.**, Single immunizing dose of recombinant adenovirus efficiently induces $CD8^+$ T cell-mediated protective immunity against malaria. *J. Immunol.* 1997. **158**: 1268–1274.

74 **Schirmbeck, R., Reimann, J., Kochanek, S. and Kreppel, F.**, The immunogenicity of adenovirus vectors limits the multispecificity of CD8 T-cell responses to vector-encoded transgenic antigens. *Mol. Ther.* 2008. **16**: 1609–1616.

75 **Percivalle, E., Cassaniti, I., Sarasini, A., Rovida, F., Adzasehoun, K. M. G., Colombini, I., Isernia, P., et al.** West Nile or Usutu Virus? A three-year follow-up of humoral and cellular response in a group of asymptomatic blood donors. *Viruses*. 2020. **12**: 157.

76 **Percivalle, E., Cambiè, G., Cassaniti, I., Nepita, E. V., Maserati, R., Ferrari, A., Martino, R. D. et al.**, Prevalence of SARS-CoV-2 specific neutralising antibodies in blood donors from the Lodi Red Zone in Lombardy, Italy, as at 06 April 2020. *Euro Surveill.* 2020. **25**: 2001031.

*Abbreviations:* **Ad**: Adenovirus · **ACE2**: angiotensin converting enzyme 2 · **AFC**: affinity antibody forming cells · **Beta-CoVs**: beta-coronaviruses · **β-ME**: beta-mercaptoethanol · **CPE**: cytopathic effect · **MERS**: Middle East Respiratory Syndrome · **SARS**: Severe Acute Respiratory Syndrome · **SARS-CoV-2**: Severe Acute Respiratory Syndrome Coronavirus 2 · **vp**: viral particles

*Full correspondence:* Dr. Andrea Gambotto, Department of Surgery, University of Pittsburgh School of Medicine, W1148 Biomedical Science Tower 200 Lothrop St. Pittsburgh, PA 15213, USA.
e-mail: gambottoa@upmc.edu

Received: 5/1/2021
Revised: 27/2/2021
Accepted: 16/3/2021
Accepted article online: 27/3/2021