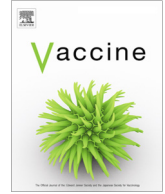Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Towards the development of an epitope-focused vaccine for SARS-CoV-2

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ABSTRACT

The rapid spread of COVID-19 on all continents and the mortality induced by SARS-CoV-2 virus, the cause of the pandemic coronavirus disease 2019 (COVID-19) has motivated an unprecedented effort for vaccine development. Inactivated viruses as well as vaccines focused on the partial or total sequence of the Spike protein using different novel platforms such us RNA, DNA, proteins, and non-replicating viral vectors have been developed. The high global need for vaccines, now and in the future, and the emergence of new variants of concern still requires development of accessible vaccines that can be adapted according to the most prevalent variants in the respective regions.

Here, we describe the immunogenic properties of a group of theoretically predicted RBD peptides to be used as the first step towards the development of an effective, safe and low-cost epitope-focused vaccine. One of the tested peptides named P5, proved to be safe and immunogenic. Subcutaneous administration of the peptide, formulated with alumina, induced high levels of specific IgG antibodies in mice and hamsters, as well as an increase of IFN-γ expression by CD8+ T cells in C57 and BALB/c mice upon *in vitro* stimulation with P5. Neutralizing titers of anti-P5 antibodies, however, were disappointingly low, a deficiency that we will attempt to resolve by the inclusion of additional immunogenic epitopes to P5. The safety and immunogenicity data reported in this study support the use of this peptide as a starting point for the design of an epitope restricted vaccine.

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1. Introduction

Vaccination has been demonstrated highly effective against SARS-CoV-2 coronavirus. However, despite all vaccines produced, there is still a need for more effective and safe vaccines against COVID-19, the cause of the worst pandemic public-health crisis in a century. Covid-19 is continuing to spread around the world, with more than 530 million confirmed cases and more than six million deaths reported across almost 200 countries. more than 63 % of people have been fully vaccinated on every continent apart from Africa (https://www.bbc.co.uk/news/world-51235105). Its rapid spread has promoted development of several vaccines in less than one year. All vaccine have targeted the full-length Spike (S) protein [1,2]. mRNA-1273 (Moderna), 2BNT162b2 (Pfizer/BioNTech), ChAdOx1-S (University of Oxford/Astra Zeneca), Gam-COVID-Vac; Sputnik V (Gamaleya Institute), Ad5-nCoV (Cansino Biological Inc), Ad26.COV2.S (Janssen Pharmaceutical/Johnson & Johnson) and NVX-CoV2373 (Novavax). All have proven effective in preventing severe COVID-19 [1,3]. The RBD domain located at C-terminal domain in S the protein, has also been considered as an effective target-vaccine [1]. RBD comprises the domain responsible for the entrance of the virus through its association to the human receptor of angiotensin-converting enzyme 2 (hACE2) [4]. Indeed, antibodies against RBD are effective to block the receptor binding [5]. Thus, RDB has been expressed under different molecular platforms [6,7,8,9,10] and as a tandem-repeat dimeric RBD protein (ZF2001) formulated with alum-based adjuvant has been already accepted for human used [11].

Despite the biotechnological success of all vaccines developed in record time, many challenges remain to be solved to overcome the COVID-19 pandemic emergency. Population in many countries still remain without access to vaccine protection, including those sectors with high probability of morbidity and mortality. The absence of immunity in a substantial portion of the world population offers the appropriate niche for the virus to evolve into new and eventually more pathogenic or more infectious variants, that can affect not only non-immune but also vaccinated individuals.

Therefore, the importance of achieving worldwide immunity as recommended by OMS against this new virus is clear, in order to interrupt transmission. Available vaccines continue to be effective against new variants of concern albeit the levels of protection have decreased. However, to compensate their decreasing efficiency, disregarding OMS recommendations, different developed countries have approved the use of additional doses of vaccine to reinforce the immunity of their populations. Favored by the lack of a global health coordination, the pandemic still poses a considerable threat. It is necessary a continued effort on vaccine development, to promote a global population immunity with an emphasis in supporting the poorest countries with more vaccines [12], as well as to become prepared against possible future changes in the virus.

Initial design relied on bioinformatic analysis of the spike protein, resulting in 6 short peptide sequences whose immunogenicity was assessed in mice. Of these, one (Peptide 5) was selected for a more extensive study on its humoral and cellular immunogenic properties. Herein, we provide the immunological characterization of the 34 amino acid peptide 5, synthetically produced, that includes crucial B-and T-cell epitopes of RBD. The information reported in this study will contribute to the development of a reproducible and secure, epitope-based vaccine.

2. Materials and methods

2.1. Mice

Mice from 9 to 16 weeks old used in this study were obtained from two different sources. Male and female BALB/c and female C57BL/6 were kindly provided by F. Rosetti from the Instituto Nacional de Ciencias Médicas y Nutrition Salvador Zubirán animal facilities. Other BALB/c mice were bred and obtained in the animal facilities of the Biomedical Research Institute. Eight to 10-week-old female and male golden Syrian hamsters were purchased from Facultad de Ciencias, UNAM animal facilities.

All procedures performed on animals in this study were conducted in accordance with national (NOM-062-ZOO-1999) and institutional regulations for the use and care of laboratory animals, following two protocols approved by the Institutional Committee (approval numbers 6343 and 6345).

2.2. Bioinformatic analysis

Epitope prediction from the RBD of the SARS-CoV-2 spike protein.

The sequence of the SARS-CoV-2 spike glycoprotein was obtained from the GenBank database (Gene ID: 43740568) (https://www.ncbi.nlm.nih.gov), and the RBD including residues 331 to 524 of the S protein [13] was analyzed for epitope prediction. We employed the Immune Epitope Database (IEDB) and IEDB-3D (https://www.iedb.org/) using the Bepipred Linear Epitope Prediction 2.0 with the scoring threshold set to 0.5 to Predict linear B cell epitopes and ElliPro: Antibody Epitope Prediction algorithms to predict discontinuous B cell epitopes using PDB ID: 7DF3 chain B, with a minimum score of 0.5 and maximum distance of 6 Å. To determine if the predicted epitopes were exposed to the protein surface and the degree of contact to ACE2, tridimensional structures of the SARS-CoV-2 S protein (PDB ID: 7DF3, and the SARS-CoV-2 S RBD/ACE2 complex (PDB ID: 7DF4) were used. The homotrimeric complex (chains A-C) of protein S was visualized and the exposed residues of the predicted epitopes were identified by calculating the solvent accessible area with Swiss-PdbViewer software [13]. In addition, the residues of the predicted epitopes exposed to ACE2 were identified knowing the polar (hydrogen bonds) and hydrophobic (van der Waals) interactions established in the cocrystallized complex RBD/ACE2 (PDB ID: 7DF4). The SARS-CoV-2 S RBD residues bound to ACE2 were identified using the Discovery Studio (https://www.3ds.com/products-services/biovia/products/molecular-modeling-simulation/biovia-discovery-studio/) software, establishing as geometric parameters of the interacting atoms, the following thresholds: 3.5 Å for hydrogen bond and 4.0 Å for hydrophobic interactions.

2.3. Peptides

Peptides were purchased from GenScript (NJ, USA) or from Synpeptide Co (Shangai, China); specifications from both manufacturers indicated >95 % purity. Peptides were dissolved at 5 mg per mL according to their physicochemical properties: peptides 1 and 2 were dissolved in 0.9 % saline solution (ISS), peptides 3 and 4 in sterile water, and peptides 5 and 6 in DMSO (1 %) and sterile water. The helper peptide was prepared at 2 mg per mL in DMSO (1 %) and sterile water.

2.4. Immunization

Groups of six to eight mice received three doses of each peptide diluted in saline together with a single adjuvant by subcutaneous (sc) or intranasal (int) routes, every 7 days as described in figures legends and tables. For s.c. vaccination, mice were immunized in the dorsal flank (27Gx13 mm needle) with a final volume of 100 μL, and for int. vaccination mice received a maximum of 12.5 μL per nostril.

Adjuvants employed were AddaVax (MF59 analog, Invivogen) or aluminum hydroxide [Al (OH)$_3$] (kindly donated by J. Garza-Ramos, PRONABIVE México). AddaVax or Al (OH)$_3$ were mixed 1:1 with each peptide to a final volume of 100 μL.

Additionally, a group of nine mice were subcutaneously administered with peptide 5 at 50 μg, plus influenza vaccine (Fluzone ®, SanofiPasteur) at 10 μg mixed with aluminum hydroxide (1:1) to a final volume of 300 μL. Mice were kept with food and water *ad libitum* and monitored daily and weighted before each immunization and sacrificed. Blood samples were taken before immunization, and 7 days after the last immunization to determine antibody levels of individual mice. The resulting sera were frozen at −20 °C until use. Broncho-alveolar fluids were also recovered 7 days after the last immunization and kept at −70 °C until use for antibody level determination.

Groups of five to six hamsters including males and females were immunized s.c. or int. with peptide 5 mixed with aluminum hydroxide or AddaVax (1:1) at a final volume of 100 μL. The animals were immunized three times at an interval of seven days, using doses of 50, 100 or 200 μg.

2.5. Antibody detection by ELISA

Serum antibody levels were determined by indirect ELISA, using the respective peptide as antigen source, following a previously described procedure with minor modifications [14]. Binding of the peptide or protein target to the wells was carried out using 100 μL of each peptide (5 μg/mL) in carbonate buffer. The plates were incubated overnight at 4 °C, washed and then incubated with 100 μL of each serum diluted 1:50 in PBS-BSA 1 % for one h at 37 °C. Antibodies were detected with 100 μL of goat anti-mouse IgG (H + L) alkaline-phosphatase conjugate (Sigma), diluted to the optimum concentration (1:5000) and followed by substrate (p-nitrophenyl phosphate, Sigma, 1 mg/mL). The reaction was stopped using 50 μL/well of NaOH 2 N, and absorbance values were determined at 405 nm in a microplate spectrophotometer reader (Epoch, Biotek, USA). Results were the average of triplicate cultures.

2.6. Cross-reactivity between anti-peptide 5 antibodies and the spike protein RBD domain

For human sera, ELISA plates were coated with Peptide 5 at 5 μg per mL in citrate buffer or with recombinant RBD expressed in eukaryotic cells (kindly provided by UDIBI, at the National Polytechnic Institute) at 1 μg per mL in carbonate buffer. Five sera from COVID-19 convalescent and 46 hospitalized patients were obtained in the Instituto Nacional de la Nutrición, Instituto Nacional de Enfermedades Respiratorias and Instituto Nacional de Neurología y Neurocirugía. Sera from healthy donors obtained in 2014 were used as negative controls. Sera were diluted in PBS containing 1 % skimmed milk, incubated for 2 h at 25 °C and, after washing 5 times, the plates were developed with horseradish-peroxidase conjugated goat anti-human IgG (1:10,000, 45 min.) and the absorbance at 450 nm was evaluated. Results were the average of triplicate determinations.

2.7. Virus neutralization assay

The assay was performed according to [15]. Fifty microliters of 2-fold serial diluted mouse sera were preincubated with an equal volume of SARS-CoV-2 (MOI = 0.1) at 37 °C for 1 h. and the mixture was then added to VERO E6 cells seeded in 96-well plates for 3 days at 37 °C and 5 % CO2. The resulting cytopathic effect (CPE) was visualized every day under the microscope. The cells were washed and fixed with ethanol:acetone (1:1) for 15 min. and finally stained

with violet crystal for 20 min. Positive and negative controls were included in the assay. Results were the average of three sera per group. This procedure was carried out in a BSL3 laboratory.

2.8. Cellular immune response

Briefly, splenocytes were obtained from peptide P5 immunized or unimmunized control mice and cultured in 24-well plates with 2 x10^6 cells per well. To evaluate the antigen-specific T cell response, cells were first Incubated with Cell Trace Violet (CTV) (Invitrogen Inc, City State), for 15 min at 37 °C, washed and then stimulated with peptide 5 (10 μg/ml), anti-CD3+ anti-CD28 antibodies (1 μg/ml) as a positive activation control or as a negative control, with RPMI complete medium alone, for three days. Cells were harvested and stained for 20 min at room temperature with anti-CD4 APC, anti-CD8 PE and with Zombie NIR, as viability dye (all from Biolegend). Next, cells were permeabilized (Tonbo Permeabilization Kit) for 1 h at room temperature and Fc receptors were blocked with anti CD16/32 (Biolegend) for 20 min at 4 °C followed by intracellular staining with anti-IFNγ BV510 (Biolegend), for 30 min 4 °C. Samples were acquired in an Atunne NxT Acoustic Focusing Flow Cytometer (Thermo Scientific) and analyzed with Flow Jo 10 software (Tree Star Inc). Results were the average of triplicate cultures.

2.9. Clinical and pathological evaluation in vaccinated and non-vaccinated mice and hamsters

To evaluate the safety of the vaccine candidate peptides, the number of deaths or apparent undesirable clinical signs were registered. Body weights of male and female immunized, and non-immunized mice and hamsters of all treatment groups were evaluated along the study period. Three mice or hamsters included in each of the treatment groups were humanly sacrificed under anesthesia on day 7 after the last immunization. The liver, heart, brain, lungs were fixed in 10 % formaldehyde and embedded in paraffin. Three μm thick serial-sections were prepared from non-consecutive areas, stained with hematoxylin and eosin (H&E), and at least two sections per tissue for each animal were examined for the presence of histological abnormalities (magnification: 400 × and 100 ×).

3. Results

3.1. Bioinformatic identification of RBD vaccine epitopes

Identification of immunogenic epitopes within RBD is essential for the development of an effective peptide vaccine against COVID-19. Six different epitopes (P1-P6) with high antigenic score were predicted between residues 407 to 506 of the RBD domain (Fig. 1). All the epitopes were highly surface exposed, but neither P2 nor P6 interact with ACE2, and P1 only had a polar interaction to ACE2 at Q42. The epitope P3 can potentially associated with ACE2 mainly with polar interactions (F490, Q493, S494, G496, T500 and Y505) and some hydrophobic interactions (Q493 and G502).

While P5, had the highest number of residues theoretically interacting with ACE2 (A475, G476, N487, F490 and Q493 via hydrogen bonds and Q474, G476, N487, F490, and Q493 via hydrophobic interactions) (Supplementary Table 1).

3.2. Antibody responses to the RBD synthetic peptides

The predicted peptides were synthetized and tested for their ability to elicit specific IgA and IgG antibodies in BALB/c and C57BL/6 mice. The mice were immunized either subcutaneous (s. c.) or intranasal, three times every-seven days and bled before the first immunization as well as seven days after the last immu-

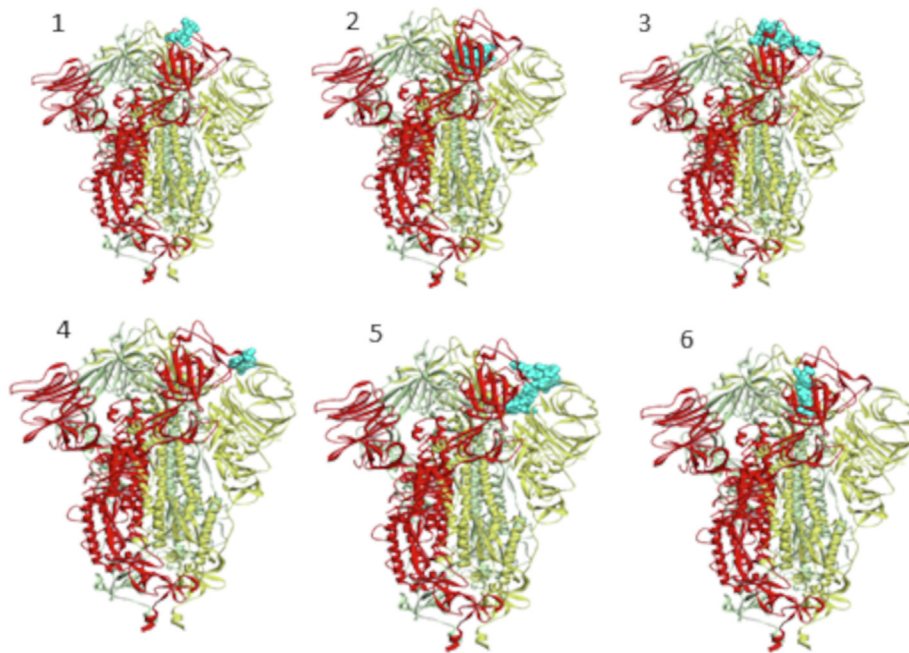| Peptide	Sequence
1	$N_{439}NLDSKVGG_{447}$
2	$V_{407}RQIAPGQTGKIAD_{420}$
3	$F_{490}PLQSYGFQPTNGVGYQ_{506}$
4	$Y_{472}QAGSTPCNGV_{483}$
5	$C_{460}LKPFERDISTEIY_{472}QAGSTPCNGV_{483}EGFNCYF_{490}PLQ_{493}$
6	$G_{431}CVIAWNS_{438}$

Fig. 1. Prediction of immunogenic peptides within RBD. Upper panel shows the amino acid sequences for peptides 1 to 6. Lower panel shows 3D homotrimeric complex of the SARS-CoV-2 S protein (PDB ID: 7DF3), each polypeptide chain is indicated in red, blue and gold. Peptides are located in the RBD and correspond to the apical region of the virus and the position of the 6 peptides are indicated by blue spheres. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

nization before being humanly sacrificed. Broncho-alveolar fluids were also recovered. For the statistical analysis, mice were grouped by body weight.

Both P4 and P5 peptides elicited a significant IgG response in s. c. vaccinated BALB/c and C57BL/6 mice. Only sera that were positive to peptide 5 recognized a recombinant RBD produced in eukaryotic cells (Table 1). Intranasal administration failed to elicit detectable levels of IgA antibodies in serum (Supplementary Table 2) and bronchoalveolar fluid (data not shown). Thus, peptide P5 was selected for subsequent analysis.

3.3. Reported mutations within peptide 5 sequence

The mutations reported worldwide in the Global Initiative on Sharing All Influenza Data (GISAID; https://www.gisaid.org) [16] for P5 were identified (Supplementary Table 3). The update shows that the entire P5 sequence has mutations; the reported occurrence allows assessing the mutational influence for each P5 amino

acid. The relevant amino acids for being part of the worldwide circulation variants are: T478K (Delta variant), E484K (Beta, Gamma and Iota variants), E484Q (Kappa variant). Also, S477N and F490S should be considered for high incidence.

Mutations were ordered according to their reported occurrence. Considering the most abundant mutation (the first on the left in each mutation), most of the substitutions were conservative mutations, corresponding to similar biochemical properties (e.g., size, polar, hydrophobic, or aromatic amino acid) compared to P5. However, when considering amino acid charges, three mutations in the polar amino acids can significantly change the electronic structure of P5, because they change from neutral to polar basic (T478K, N481K) and from acidic to basic (E484K) (Supplementary Table 3).

3.4. Differential antibody responses between mice strains immunized with peptide P5

To define the optimal P5 dose inducing the highest specific antibody response, female C57BL/6 and BALB/c mice were immunized

Table 1
Levels of sera IgG and IgA in mice subcutaneously immunized with different peptides derived from the RBD domain of the Spike protein of SARS-CoV-2 virus.

	Sera levels of IgG				Sera levels of IgA	
	Recognizing the specific peptide		Recognizing the RBD domain		Recognizing the specific peptide	
	Before Immunization	After	Before Immunization	After	Before Immunization	After
Immunizing with:						
P1	0.20 ± 0.02	0.19 ± 0.04	0.26 ± 0.09	0.24 ± 0.02	0.16 ± 0.10	0.22 ± 0.11
P2	0.25 ± 0.06	0.17 ± 0.01	0.19 ± 0.03	0.23 ± 0.01	0.09 ± 0.02	0.17 ± 0.05
P3	0.25 ± 0.12	0.25 ± 0.10	0.24 ± 0.10	0.24 ± 0.09	0.09 ± 0.02	0.18 ± 0.09
P4	0.44 ± 0.07	1.69 ± 1.60*	0.16 ± 0.08	0.28 ± 0.04	0.08 ± 0.03	0.20 ± 0.08
P5	0.22 ± 0.02	1.10 ± 0.34*	0.23 ± 0.06	2.21 ± 1.38*	0.17 ± 0.00	0.17 ± 0.09
P6	0.14 ± 0.02	0.14 ± 0.05	ND	ND	0.29 ± 0.09	0.45 ± 0.31

Mean ± SD of the Optical Density read at 405 nm of the ELISA to detect IgG and IgA level of immunoglobulins of six female mice subcutaneously immunized with 50 μg of the respective peptide with ADDAVAX. Each mouse received three doses of the vaccine each 7 days. Mice were bled before and seven days after the last immunization. ND: not determined. *Statistically different between before and after immunization (P < 0.05).

3 times. Similarly, high levels of IgG were observed in C57BL/6 after s.c. immunization with all doses of P5. In contrast, there was a dose dependent antibody response to P5 in BALB/c mice (Table 2). Thus, subcutaneous immunization induced high antibody responses in both strains of mice, with a higher antibody response at the lower dose of P5 in C57BL/6 mice, the most inflammatory strain. Furthermore, intranasally immunized mice elicited low but significant IgG specific antibody levels in C57BL/6 mice for all doses used. Finally, insignificant levels of anti-P5 IgA antibodies were detected in sera from s.c. or intranasally immunized mice (Supplementary Table 4).

3.5. Sexual dimorphism in the antibody response induced in peptide P5 immunized mice

Initial experiments pointed to the existence of sexual dimorphisms in the antibody levels induced by peptide P5 in mice (data not shown). This was further explored by immunizing female and male BALB/c mice using the same protocol described above. As shown in Supplementary Table 5, significantly higher levels of specific IgG antibodies were induced in female than in male mice after s.c. immunization with 3 doses of peptide P5.

3.6. Aluminum hydroxide is more effective adjuvant than AddaVax for vaccination of mice

To evaluate the best formulation for the vaccine, we performed experiments employing an oil-based adjuvant (AddaVax) or aluminum hydroxide (Aluminatrihydrate). Both adjuvants are approved for use in humans. Production of specific IgG anti-P5

antibodies was similar when mice were immunized s.c. using either adjuvant. Higher levels of antibodies were found in C57BL/6 mice relative to BALB/c, independent of the adjuvant used (Table 3). Low levels of specific IgA antibodies were detected in broncho-alveolar fluids of mice after s.c. and intranasal administration (Supplementary Table 6), but neither adjuvant induced detectable levels of IgA antibodies in serum (data not-shown).

3.7. Aluminum hydroxide was more effective than AddaVax for the induction of anti-P5 IgG antibodies in hamsters

Considering that hamsters are natural hosts for SARS-CoV-2, the immunogenicity of P5 was also evaluated in hamsters. High levels of specific IgG antibodies were induced by s.c. immunization of hamsters with 3 doses of P5 using AddaVax as adjuvant and they were dose dependent for P5 (Table 4). However, higher levels of specific IgG antibodies were induced in hamsters immunized with the lower dose of P5 using aluminum hydroxide as adjuvant. No sexual dimorphism in the antibody response was observed in hamsters under these immunization protocols. Finally, no IgG levels were detected in the sera after intranasal immunization of hamsters with P5 (Table 4).

3.8. No clinical and pathological features were associated with peptide P5 immunization

No deaths or apparent clinical signs were found in any group of mice or hamsters treated with 3 doses of P5 in combination with either of the two adjuvants. The weight of both species of animals remained statistically unchanged, before and after immunization

Table 2
Effect of different doses of P5 on the level of specific antibodies in Intranasal and Subcutaneously immunized mice.

		Anti-P5 IgG antibodies					
		Subcutaneous			Intranasal		
	P5 (μg/mouse)	Before Immunization	After	P5 (μg/mouse)	Before Immunization	After	
Mouse Strain:							
C57Bl/6J	50	0.25 ± 0.04	2.72 ± 0.01*	10	0.25 ± 0.05	0.41 ± 0.10*	
BALB/c	50	0.21 ± 0.01	0.32 ± 0.01*	10	0.27 ± 0.08	0.29 ± 0.02	
C57Bl/6J	100	0.16 ± 0.01	2.90 ± 0.03*	50	0.38 ± 0.16	0.55 ± 0.02*	
BALB/c	100	0.30 ± 0.01	1.43 ± 1.20*	50	0.21 ± 0.01	0.29 ± 0.07	
C57Bl/6J	200	0.31 ± 0.01	2.09 ± 0.80*	100	0.26 ± 0.04	0.37 ± 0.10*	
BALB/c	200	0.33 ± 0.02	2.82 ± 0.06*	100	0.21 ± 0.08	0.19 ± 0.01	

Mean ± SD of the Optical Density read at 405 nm of ELISA to detect levels of IgG anti-P5 antibodies in female mice immunized with different doses of P5 adjuvanted with Addavax. Each mouse received three doses of the vaccine each 7 days. Mice were bled before and seven days after the last immunization. *Statistically different between before and after immunization (P < 0.05).

Table 3
The optimal humoral immune response was induced by subcutaneous immunization with peptide 5 and aluminum hydroxide.

			Anti-peptide 5 IgG	
			Before Immunization	After
	Mouse Strain:	Adjuvant		
Subcutaneous immunization	C57BL/6J	ADDAVAX	0.21 ± 0.01	2.58 ± 0.20*
	BALB/c	ADDAVAX	0.25 ± 0.04	0.32 ± 0.02
	C57BL/6J	AL(OH)3	0.26 ± 0.06	2.68 ± 0.28*
	BALB/c	AL(OH)3	0.16 ± 0.03	0.81 ± 0.54*
Intranasal Immunization	C57BL/6J	ADDAVAX	0.25 ± 0.05	0.37 ± 0.10
	BALB/c	ADDAVAX	0.27 ± 0.07	0.19 ± 0.01
	C57BL/6J	AL(OH)3	0.26 ± 0.06	0.36 ± 0.14
	BALB/c	AL(OH)3	0.16 ± 0.06	0.17 ± 0.02

Mean ± SD of the Optical Density read at 405 nm of ELISA to detect sera levels of IgG anti-P5 antibodies from female mice immunized with P5 adjuvanted either with Aluminum hydroxide or ADDAVAX. Each mouse received three doses of the vaccine each 7 days. Mice were bled and sacrificed seven days after the last immunization. The level of IgG antibodies in the group in separates groups of two BALB/C, C57BL/6J or non– immunized mice, were determined. *Statistically different between before and after immunization (P < 0.05).

Table 4
Effect of adjuvant and dose of P5 on the levels of IgG antibodies in subcutaneous and intranasal immunized female and male hamsters.

				Sera IgG levels				
				Before Immunization	After	Route	Before Immunization	After
Adjuvanted with: ADDAVAX	Sex	Dose (µg)	Route					
	M	10	SC	ND	ND	IN	0.16 ± 0.01	0.17 ± 0.01
	F							0.21 ± 0.07
	M	50	SC	0.19 ± 0.02	1.45 ± 0.09*	IN	0.15 ± 0.01	0.20 ± 0.08
	F				1.42 ± 0.45*			0.29 ± 0.09
	M	100	SC	0.13 ± 0.03	3.45 ± 0.35*	IN	0.14 ± 0.01	0.17 ± 0.04
	F				3.72 ± 0.07*			0.29 ± 0.09
	M	200	SC	0.13 ± 0.01	3.36 ± 0.34*	IN	ND	ND
	F				3.59 ± 0.06*			
Aluminum hydroxide	M	50	SC	0.19 ± 0.03	3.76	IN	ND	ND
	F				3.06 ± 0.49*			
	M	100	SC	ND	ND	IN	0.15 ± 0.01	0.20 ± 0.03
	F							0.26 ± 0.01

Mean ± SD of the Optical Density read at 405 nm of ELISA to detect sera levels of IgG anti-P5 in male and female hamsters immunized with different doses of P5 with either ADDAVAX or AL(OH)$_3$. Each hamster received three doses of the vaccine each 7 days. A pooled serum from a group of non-immunized hamsters was employed and samples from each immunized hamster was obtained seven days after the last immunization in those immunized. *Statistically different between before and after immunization (P < 0.05). ND: No determined.

with P5 (Suppl. Fig. 1). Furthermore, after histological inspection of control and immunized mice, no abnormalities were found in the brains, kidneys, spleen, pancreas, heart, small and large intestines (data no shown). The most important hepatic alteration was vacuolar degeneration that occurred in 100 % of the samples and in mild to moderate grades (Suppl. Fig. 2 A, C). Some hepatocytes were found with prominent karyomegaly and nuclei, and pyknotic nuclei in two individuals. These findings were also found in the control group, so they are not associated with treatment. In the lung. The most important alteration was alveolar macrophage hyperplasia, which occurred in 80 % of individuals and was associated with antigenic stimulation independent of treatment (Suppl. Fig. 2 B, D). As observed in mice no histological abnormalities were found in hamsters (data not shown).

3.9. Immunogenicity of peptide P5 vs RBD

In order to compare the relative effects of s.c. immunization using peptide P5 and recombinant RBD, specific antibody levels were determined at different time points after 3 immunizations with each antigen. As shown in Fig. 2A, similar levels of IgG anti-

bodies were induced by peptide P5 and RBD and remained detectable up to 60 days after the last immunization.

3.10. Anti-peptide P5 antibody virus neutralization assay

The capacity of antibodies elicited by P5 peptide immunization to interfere with the entrance of SARS-CoV-2 virus to the cells was measured. BALB/c mice were immunized three times on days 0, 7 and 14 with 50 µg of peptide P5 mixed with Al(OH)$_3$. Sera from three mice per group was collected on day 14, 21 and 44 days after the first immunization and serial dilutions were incubated with the SARS-CoV-2 virus and VERO E6 cells. Immunization significantly increased neutralization antibody titers (4 ± 0 and 4.74 ± 0.58 at 21 and 44 days, respectively), whilst only a tendency was obtained after the second immunization, as shown in Fig. 2B.

3.11. Antibodies in sera from COVID-19 patients and convalescent individuals recognize peptide P5

Recent studies have demonstrated that sera from infected patients can recognize the RBD domain of SARS-CoV-2 [17]. To test if SARS-CoV-2 infection induces antibodies that also recognize P5,
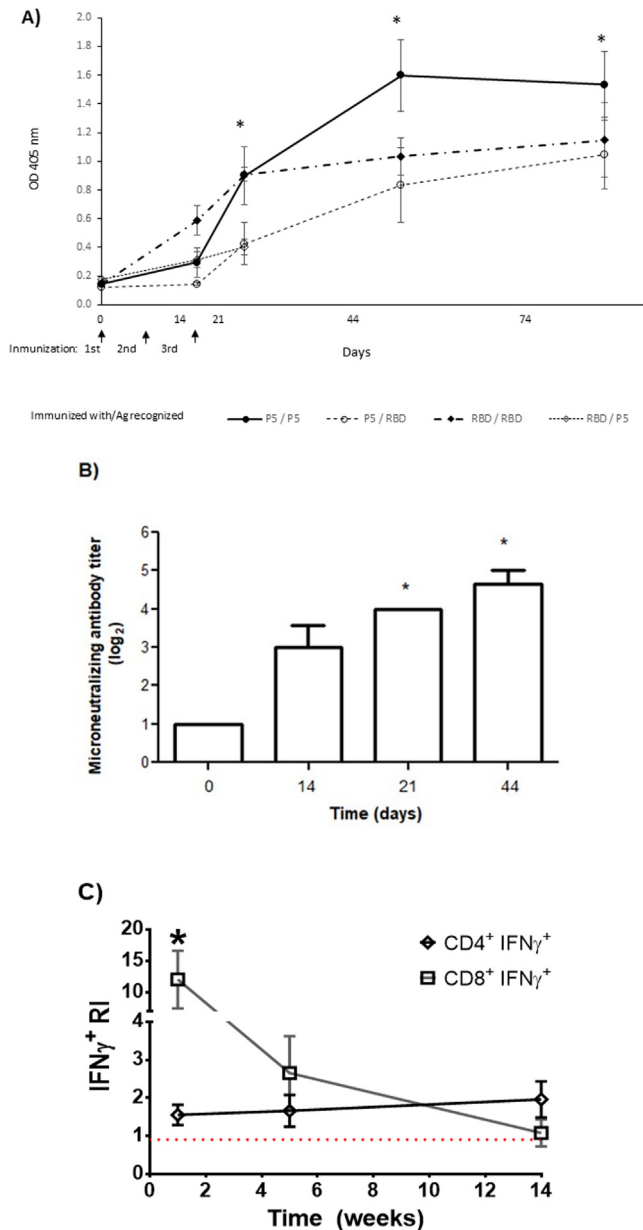
Fig. 2. Humoral and cellular immune response sustained. Antibodies induced by s. c. immunization with P5 or RBD. **A)** Subcutaneous immunization of mice was carried out in groups of six to twelve BALB/c mice female mice, using 50 μg of peptide 5 or 12.5 μg of RBD, in the presence of alumina. Each mouse received three doses of peptide 5 or RBD each 7 days. anti-peptide 5 or anti-RBD IgG antibodies were determined at the indicated time points by ELISA. Mean + SEM are shown and statistical differences are depicted as *P < 0.05. **B)** Microneutralizing antibody (MN) titers against SARS-CoV-2 virus. Mice were immunized s.c. three times at weekly intervals with P5, and sera were obtained at different time points. Neutralization of SARS-CoV-2 infection (MOI = 0.1) by individual diluted sera (n = 3) from mice was determined. Sera from convalescent patient were used as controls. Solid bars represent geometric mean log2 titer with SEM shown by error bars. Statistically different between before and after immunization (*P < 0.05). **C)** Splenocytes from BALB/c mice s.c. immunized with P5 adjuvanted either with Aluminum hydroxide were recovered at weeks 1, 5 or 14 after the last immunization. Cells were restimulated *in vitro* with P5 (10 μg/ml), anti-CD3 + anti-CD28 antibodies (1 μg/ml) as positive control or medium alone (W/S) for 72 h. The mean and SD of the intracellular IFNγ production of CD4+ and CD8+ T cell subpopulations, were plotted. Relative index (RI) was calculated with respect to non-immunized mice.

46 sera from infected individuals were obtained from hospitals treating COVID-19 patients. All sera from convalescent individuals and 89 % of sera from COVID-19 patients recognized peptide P5 and RBD (Fig. 3). This agrees with our observation that about half

of the sera from mice immunized with P5 induced antibodies that react with both, P5 and RBD. (Fig. 3).

3.12. Immunization of mice with human influenza vaccine reduced the antibody response to peptide P5

As the influenza vaccine may be administered in combination with a COVID-19 vaccine, particularly during future cold seasons, the effect of co-immunization of mice with peptide P5 plus human influenza vaccine was explored. Co immunization with influenza vaccine and the peptide reduced the antibody response to peptide P5, but not to the influenza vaccine (Suppl. Fig. 3).

3.13. Cellular immune response to the RBD synthetic peptide P5

To evaluate antigen specific T cell responses, splenocytes from 3 times immunized and unimmunized mice were re-stimulated *in vitro* for 72 h with peptide P5 (10 μg/ml). Intracellular IFNγ production of CD4+ and CD8+ T cell subpopulations was analyzed by flow cytometry (Fig. 4). Our results showed that significant IFNγ production was observed only in CD8+ T cells from mice that were s.c. immunized using aluminum hydroxide as adjuvant (Table 5).

To evaluate the kinetic of the cellular response along the time, splenocytes from peptide P5 immunized mice were tested at different times after 3 immunizations schedule with P5-aluminum hydroxide. As shown in Fig. 2C, whilst the number of CD4+ T cells secreting IFNγ was only slightly increased during the evaluation period, the number of IFNγ secreting CD8+ T cells was robustly increased after a week, and then gradually decreased to similar levels as the CD4+ T cells.

Taken together, these findings reinforce our decision to use aluminum hydroxide as the optimal adjuvant to induce both, a specific humoral response, as well as an IFNγ producing T cell response.

4. Discussion

Several vaccines have been developed against COVID-19 at an unprecedented rate and billions of doses have been administered worldwide, dramatically lowering deaths from this disease. However, despite great international efforts, the pandemic is far from over. Although vaccines available effectively reduced severe COVID-19 cases, they are less effective in reducing transmission [18]. The latter together with the relaxation of health containment measures, have resulted in the continued transmission of SARS-CoV-2 in large numbers of infected individuals, favoring the emergence of new variants that are progressively distinct from the original strain for which the current vaccines were designed [19,20]. Moreover, reaching global vaccination coverage remains a major challenge, particularly in lower-income countries. Thus, it is highly relevant to continue with the research and development of vaccines using platforms with the potential to increase global production of effective vaccines.

The S protein of SARS-CoV-2 is the major target of most anti-COVID-19 vaccines currently available. In this study, *in silico* predictions of immunogenicity were used for a rational design of multi-epitope peptide-based vaccines. Immunogenic properties of 6 peptides predicted by *in silico* analysis of the S protein were tested through immunization of mice. A peptide of 34 aa named P5, induced the highest levels of specific IgG antibodies in two strains of mice, as well as in hamsters. Differences in the magnitude of the responses were observed depending on the route of administration, dose, number of doses, adjuvants employed in vaccine formulation, animal species, as well as strain and gender in mice.
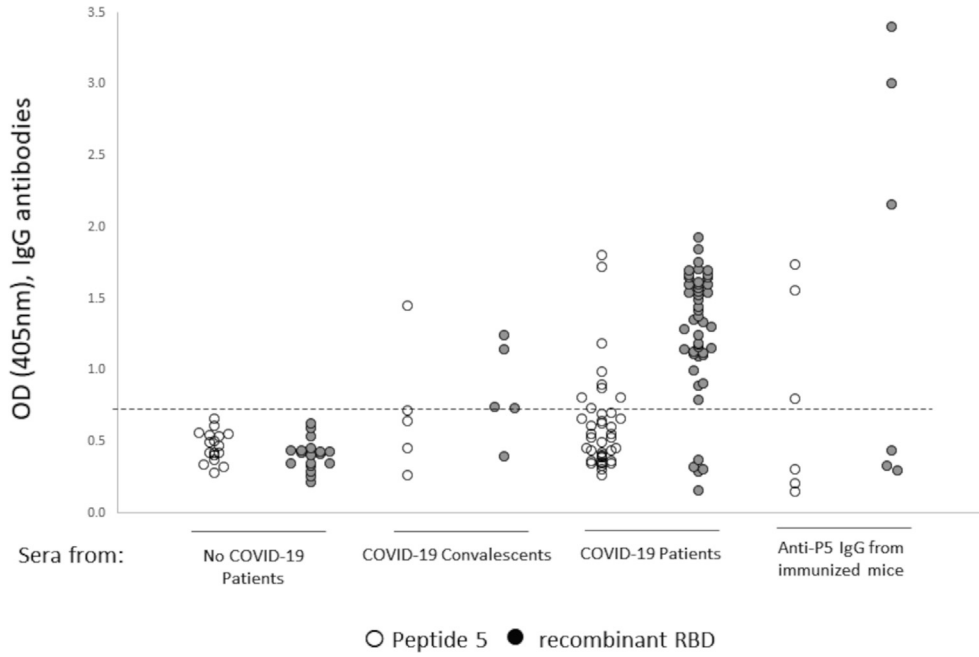
Fig. 3. Cross-reactivity between peptide 5 and RBD. IgG antibodies against peptide 5 or RBD were determined in sera from six COVID-19 recovered-convalescents subjects, 20 other patients with active COVID-19, and from 6 mice immunized with Peptide 5 as described above. Data are expressed as the mean ± SD of the IgG levels that recognized peptide 5 or the recombinant RBD.
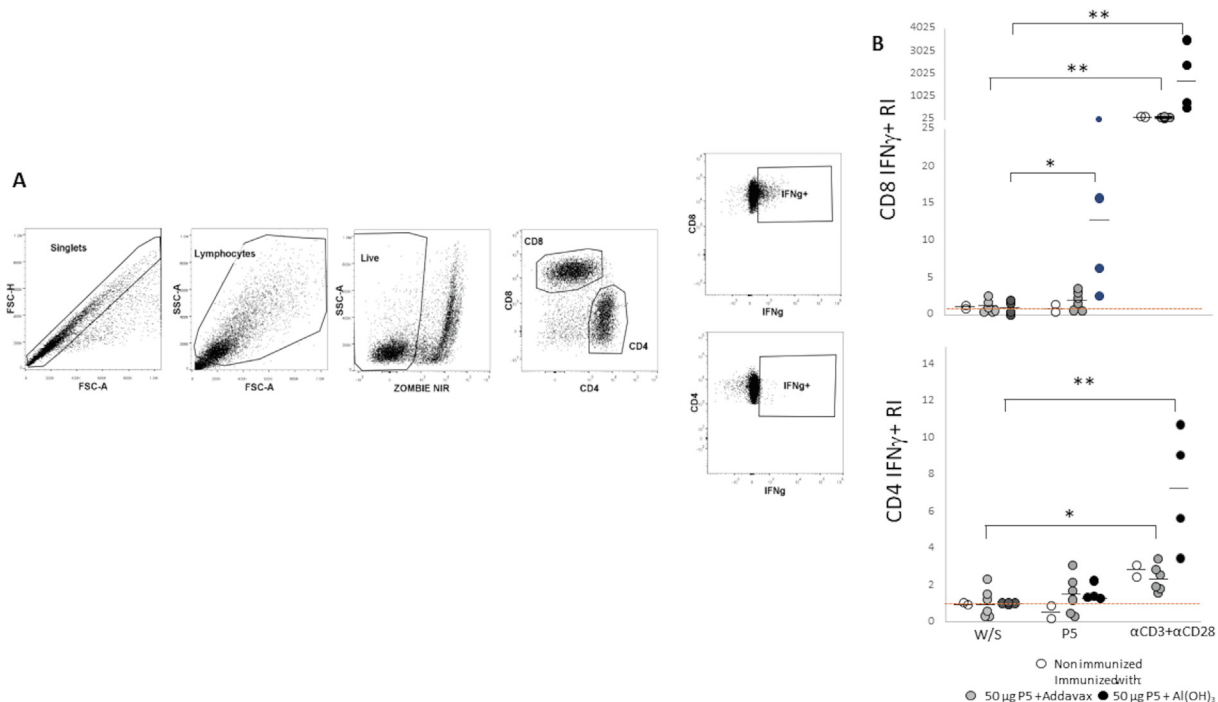

Fig. 4. Cellular immune response induced by P5. Splenocytes from BALB/c mice s.c. immunized with P5 adjuvanted either with Aluminum hydroxide or ADDAVAX were recovered 7 days after the last immunization. Cells were restimulated *in vitro* with P5 (10 μg/ml), anti-CD3 + anti-CD28 antibodies (1 μg/ml) as positive control or medium alone (W/S) for 72 h. **A)** Graphs show the gating strategy used to analyze CD4+ and CD8+ T cells. **B)** Intracellular IFNγ production of CD4+ and CD8+ T cell subpopulations was analyzed by flow cytometry. *Statistically different between before and after immunization (P < 0.05).

Peptide P5 induced strong humoral and cellular immune responses when subcutaneously administered to both C57BL/6 and BALB/c mice. Similar responses were produced in hamsters. The lower dose of peptide was the most effective for the stimulation of both humoral and cellular responses. Although most of the experiments reported here were performed using 3 immuniza-

tions, mice also elicited a significant antibody response after the second dose of antigen. Indeed, detectable antibodies induced by two subcutaneous immunizations of P5 using aluminum hydroxide as adjuvant elicited both ELISA positive and virus neutralizing antibodies, which were maintained for at least 80 days after the last immunization, suggesting that peptide P5 includes relevant B-

Table 5
The optimal cellular immune response was induced by subcutaneous immunization with peptide 5 and aluminum hydroxide.

			Cellular immune response (CD8 + IFNγ + cells)	
			Before Immunization	After
	Mouse Strain:	Adjuvant		
Subcutaneous immunization	C57BL/6J	ADDAVAX	ND	0.46 ± 0.47
	BALB/c	ADDAVAX	0.86 ± 0.68	0.76 ± 0.03
	C57BL/6J	AL(OH)$_3$	ND	4.4 ± 2.6*
	BALB/c	AL(OH)$_3$	0.86 ± 0.68	20.83 ± 7.3*
Intranasal Immunization	C57BL/6J	ADDAVAX	ND	0.58 ± 0.05
	BALB/c	ADDAVAX	0.86 ± 0.68	1.34 ± 0.6
	C57BL/6J	AL(OH)$_3$	ND	0.60 ± 0.08
	BALB/c	AL(OH)$_3$	0.86 ± 0.68	0.75 ± 0.40

Mean ± SD of the number of spleen CD8 + IFNγ + cells from female mice immunized with P5 adjuvanted either with Aluminum hydroxide or ADDAVAX. Each mouse received three doses of the vaccine each 7 days. Mice were bled and sacrificed seven days after the last immunization. The numbers of CD8 + IFNγ + cells in separates groups of two BALB/C, C57BL/6J or non- immunized mice, were determined. *Statistically different between before and after immunization (P < 0.05). ND: No determined.

cell epitope(s) for the induction of a sustained and effective humoral and cellular response.

Neutralizing antibodies titers of vaccinated mice ranged 4 to 4.74O.D. Meanwhile, the sera of the individuals vaccinated against Covid-19 presented a range of neutralization titers from 2 to 10O. D. We can therefore consider that titers of neutralizing antibodies of P5 vaccinated mice are in the range of the least responsive individuals to the vaccine.

As in many viral infections, cellular immunity plays an important role in resistance to SARS-CoV2 [21]. Indeed, an inverse association has been reported in COVID-19 between the severity of the disease and the percent of activated and proliferating virus-specific CD8+ T cells, such as spike-specific CD8+ T cells [22]. Thus, the observation that peptide P5 induced a proliferative response of CD8+/IFNγ mouse cells is particularly significant. The T cell response was evaluated when P5 was administered with aluminum hydroxide as adjuvant, but not with AddaVax (Table 5). This is of particular practical relevance as aluminum salts have been used for the formulation of vaccines for almost 100 years. In addition to its safety, the low cost and lack of intellectual property claims makes aluminum hydroxide an ideal adjuvant for development of vaccines, particularly in developing countries.

An interesting sexual dimorphism in the antibody response of mice was also observed; being females more responsive than males. Gender differences in favor of women in the risk of contracting and dying from COVID-19 have been documented in different countries and similar data were observed in recent epidemics, including severe acute respiratory syndrome (SARS) and in the Middle East respiratory syndrome (MERS) [23,24]. The mechanisms underlying sex differences in vaccine-induced innate and adaptive immunity has implicated hormonal, genetic, and even microbiota differences between males and females [25]. Generally, testosterone has an immunosuppressive effect while estrogen has an immunoenhancing effect on the immune system [26,27]. The X chromosome may be also involved in the sex-related immune function [28]. Thus, a dimorphic response to vaccine-induced humoral immunity similar to that reported here for peptide P5 immunized mice seems to be frequent, and has been described for other vaccines [25,29,30,31].

Another point that merit comments is the lower immunogenicity observed when P5 was intranasally administered. Clearly, increasing mucosal immunity to control respiratory virus infection is desirable. Despite this consideration and the multiple worldwide efforts for the development of intranasal vaccines, all the vaccines initially developed all designed for intramuscular immunization. Until this year, intranasal vaccines have begun to be licensed, reflecting a major challenge in inducing protection by this route [32]. It is possible that the formulation used herein for P5 was not the optimal to achieve the immunity induced by the injectable version.

An important consideration for COVID-19 vaccines is the occurrence of aminoacids changes in the spike protein. Supplementary Table 3 shows the substitution, deletions, and mutations according to the information reported in September 13, 2022 (https://gisaid. org/hcov19-variants/) The substitutions with the highest occurrence frequencies were: T478K, S477N, and E484A reported in Omicron GRA and Delta GK variants. E484K (0.2x106) in Gamma GR/501Y.V3 and Beta GH. Finally, Q493R and F486V in Omicron GRA. Mutations with occurrence frequencies ≥ 500 are indicated in bold. In view of this high number of mutations on this 34 aa-peptide we considered extending the peptide to incorporate new epitopes and increase its immunogenicity using the full domain of RBD.

Finally, we have addressed the possibility of vaccine induced immunopathology, due to acute pro-inflammatory components in the vaccine or in its adjuvants. In this respect, peptide P5 did not induce any behavioral or macroscopic evidence of discomfort in the animals. In addition, there were no significant histological differences between the organs of vaccinated and control mice. Some microscopic findings observed during necropsies, were not correlated with the treatment and are probably due to the maintenance conditions in our animal facilities.

In conclusion, this study shows that a 34 amino acids peptide, included in the RBD domain, has interesting immunogenic properties for the development of a vaccine against SARS-CoV2.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2022.09.059.

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