Toxicity and Local Tolerance of a Novel Spike Protein RBD Vaccine Against SARS-CoV-2, Produced Using the C1 *Thermothelomyces Heterothallica* Protein Expression Platform

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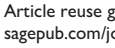

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Abstract

Coronavirus disease 2019 (COVID-19) has caused the ongoing COVID-19 pandemic and there is a growing demand for safe and effective vaccines. The thermophilic *Thermothelomyces heterothallica* filamentous fungal host, C1-cell, can be utilized as an expression platform for the rapid production of large quantities of antigens for developing vaccines. The aim of this study was to evaluate the local tolerance and the systemic toxicity of a C1-cell expressed receptor-binding domain (C1-RBD) vaccine, following repeated weekly intramuscular injections (total of 4 administrations), in New Zealand White rabbits. The animals were sacrificed either 3 days or 3 weeks following the last dose. No signs of toxicity were observed, including no injection site reactions. ELISA studies revealed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-specific immunoglobulin G antibodies in the sera of C1-RBD-treated animals starting from day 13 post injection, that were further elevated. Histopathology evaluation and immunohistochemical staining revealed follicular hyperplasia, consisting of B-cell type, in the spleen and inguinal lymph nodes of the treated animals that were sustained throughout the recovery phase. No local or systemic toxicity was observed. In conclusion, the SARS-CoV-2 C1-RBD vaccine candidate demonstrated an excellent safety profile and a lasting immunogenic response against receptor-binding domain, thus supporting its further development for use in humans.

Keywords

vaccine, rabbits, safety, toxicity, COVID-19, C1, *Thermothelomyces heterothallica*, RBD, SARS-CoV-2.

Introduction

The coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is an ongoing pandemic, which has resulted in close to 5 million deaths worldwide until now.[1,2] Vaccination is considered the most effective way to control and prevent the pandemic, and there is an increasing demand for the development of safe, effective, protective, and affordable vaccines against this condition.[3-6] Furthermore, to combat a global pandemic, it is necessary to utilize all available vaccine production platforms to ensure continuous and quick availability of vaccines to all the countries in the world, including middle-income and low-income countries.

The SARS-CoV-2 spike surface glycoprotein (S) mediates virus attachment and entry into host cells and is the sole target of neutralizing antibodies. The most effective SARS-CoV-2 neutralizing antibodies described to date target the ~25 kDa receptor-binding domain (RBD) within the S1 subunit of the S protein, as was shown for Middle East respiratory syndrome coronavirus (MERS-CoV).[7] In addition, the RBD contains one or several N-linked glycosylation sites, which likely play a role in protein folding and immune evasion.[8] More recently, a vaccine candidate based on residues 319 to 545 of the RBD, ZF2001 has received emergency use authorization in both China and Uzbekistan since March 2021 and is involved in a three-dose vaccination regimen.[9,10] Based on those records, the

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RBD was selected as an antigen for vaccination against the SARS-CoV-2 in this study.[11]

One unique expression platform is the thermophilic fungal host C1 (*Thermothelomyces heterothallica* formerly named *Myceliophthora thermophila*), which was first discovered in 1992 to be a natural neutral cellulase producer.[12] Since 2016 C1 is explicitly being used for the rapid development and production of therapeutic proteins and vaccines.[13,14] Characterization of the glycoprotein structure of the recombinant RBD-C-tag$^{333\text{-}527}$ produced in C1 has been previously compared with RBD produced in Yeast, *E. coli*, and Mammalian cells using buffer-free protein digestion with electrospray ionization-mass spectrometry analysis.[11] This confirmed its identity with the typical heterogeneity of N-glycans and the expected molecular masses, as well as the integrity of the N-terminal and C-terminal ends and formation of the four disulfide bonds. It is important to note that the C1-produced RBD-C-tag recombinant subunit antigen specifically and strongly binds hACE2 receptors and, thus possesses RBD-specific antigen epitope conformation(s) functional for hACE2 receptor binding, and therefore, has been also utilized for the production of the SARS-CoV-2 S protein RBD as a potential vaccine candidate.[15]

One of the most important steps in vaccine development is preclinical safety assessment in animals. This aims to predict the safety of the vaccine's clinical use in humans, to decrease the chance for adverse effects in clinical trial participants, and to provide information for vaccination protocols.[3,16-18] Therefore, the aim of this study was to evaluate the systemic toxicity and local tolerance of the C1-cell expressed receptor-binding domain (C1-RBD) SARS-CoV-2 vaccine, bound to aluminum hydroxide (Alhydrogel® '85'), following repeated intramuscular (IM) injections, in New Zealand White (NZW) rabbits.

Materials and Methods

Recombinant Protein Expression

A DNA sequence coding for C1 endogenous CBH1 signal sequence, residues 333 to 527 of the Spike (S1) glycoprotein from SARS-CoV-2 Spike S1 (GenBank No.: QHD43416.1), a Gly-Ser-linker dipeptide, and the Carboxy-terminal tetrapeptide C-tag (E-P-E-A) flanked by homologous recombination sequences to the C1-cell DNA expression vector and MssI restriction enzyme sites was designed and synthesized by GenScript (Piscataway, New Jersey, USA). The codon usage was optimized for expression in *T. heterothallica* and the construct was cloned into the PacI restriction site of the C1-cell expression vector plasmid, pMYT1055 under the endogenous C1*bgl8* DNA promoter to generate an expression ready DND vector.

The expression vector was transformed into C1 DNL-155 strain as described and cell clones producing RBD-C-tag protein were selected.[14]

Fermentation Development and Production of the Vaccine Candidate in 10-L Fermenters

For fermentation initiation, Petri dishes which had been inoculated with frozen mycelium were incubated at 37°C for 2 days. Next, the mycelium was scraped using a sterile cotton swab and transferred to a 250-ml shake flask containing 45 ml of growth medium, incubated at 37°C, 250 rpm, for a further 2 days. Next, 25 ml were transferred into each of two 3.5-L conical baffled Shake flasks containing 300 ml growth medium: yeast extract, 5 g/L; $(NH_4)_2SO_4$, 4.62 g/L; NaCl, 0.41 g/L; KH_2PO_4, 7.48 g/L; Casamino acids, 1 g/L after autoclaving 20 ml of glucose 50%; 2 ml of 1M $MgSO_4$; 1 ml, 1000× trace elements; and 1 ml, Spectinomycin, 150 mg/ml. These flask cultures were incubated at 37°C, 250 rpm for 24 hours.

C1-RBD was manufactured in a 10-L Bioflo 120 Fermenter (Eppendorf, Hamburg). Five liters of high concentration yeast extract medium with glucose[14] was inoculated with a seed culture of 500 ml the C1 DNL-155 production strain, Fermentation conditions were as decribed.[14]

Purification of C-RBD Antigen Using CaptureSelect™ C-Tag Affinity Matrix

Supernatants from fermentation of C1-RBD production strain were centrifuged at 32,000 ×*g*, 10 minutes, 4°C, two times, using a Sorvall RC-5C centrifuge, and filtered through a Sartopore 0.2-µm filter capsule (Sartorius AG, Goettingen, Germany). The clarified fermentation fluid was concentrated 3-fold and the buffer was exchanged with PBS, pH 7.2. For rapid purification, CaptureSelect™ C-Tag Affinity Matrix (Thermo Fisher Scientific, Waltham, MA, USA) was used to combine the unique selectivity for the 4-amino acid peptide tag (E-P-E-A). The ultra-filtrated culture supernatant containing RBD-C-tag was added to 1 ml resin and gently mixed for 10 minutes and packed in a Poly-Prep® Chromatography column (Bio-Rad, Hercules, CA, USA) using gravity. The column was washed with 10 Column Volumes of PBS, pH 7.2. The RBD-C-tag was eluted with 3 Column Volumes of buffer containing final concentrations of 20 mM Tris-HCl, pH 7.0, and 2M $MgCl_2$. The eluate contains C1-RBD with purity over 95%.

C1-RBD Antigen Vaccine Candidate Preparation and C1-RBD Alhydrogel® '85' Binding

To prepare the C1-RBD antigen vaccine candidate, 3.5 ml PBS containing 290 µg/ml C1-RBD was added to 6.5 ml of buffered saline containing 2.5 ml of Alhydrogel® '85' 2% (Brenntag Biosector A/S, Ballerup, Hovedstaden, Denmark). The aluminum content of Alhydrogel '85' is 10 mg/ml so the formulated C1-RBD contained in 200 µl dose injected to a mouse, 20.3 µg of RBD and 500 µg of Alum, respectively. The C1-RBD Alhydrogel® '85' slurry was gently mixed for 8 hours at 4°C to ensure binding of C1-RBD to Alhydrogel® '85' reached equilibrium.

Table 1. Experimental design.

		Treatment			
Group No.	No. of animals	Test material	Dose	Route and frequency	Scheduled sacrifice (days post first dosing session)
1M	5	C1-RBD placebo (control item)	0	0.25 mL/site × 2 sites/ animal, IM injections to the thigh muscles, 4 repeated injections at 1-week interval	24 days
	5				42 days
2M	5	C1-RBD vaccine (test item)	33 µg		24 days
	5				42 days
1F	5	C1-RBD placebo (control item)	0		24 days
	5				42 days
2F	5	C1-RBD vaccine (test item)	33 µg		24 days
	5				42 days

Abbreviations: IM, intramuscular; C1-RBD, C1-cell expressed receptor-binding domain.

Current Good Manufacturing Practice (cGMP) Production of the C1-RBD Drug Substance (DS)

The C1 DNL-155 RBD producing strain was run in a 20-L fermenter for 4 days in fed-batch fermentation at Bio-Technology General (BTG, Kiryat Malachi, Israel), a Ferring company, Israel as described above, under cGMP conditions. Downstream processing included, filtration, using UF 100K and 300K disposable crossflow cassettes, and CaptureSelect™ (Thermo Fisher Scientific, Waltham, MA, USA) C-tag affinity purification.[19-21]

Production of the Drug Product (DP)

The DP formulation contains 33 µg purified C1-RBD per human dose in 0.5 ml buffer with aluminum hydroxide as the adjuvant (Alhydrogel® '85' 2.0%). The total aluminum amount was 1.2125 mg/dose. The placebo contained only aluminum hydroxide (Alhydrogel® '85' 2.0%) in buffer. The C1-produced SARS-CoV-2 RBD vaccine candidate was supplied in 10-ml borosilicate glass vials that were stored at 2°C to 8°C before use.

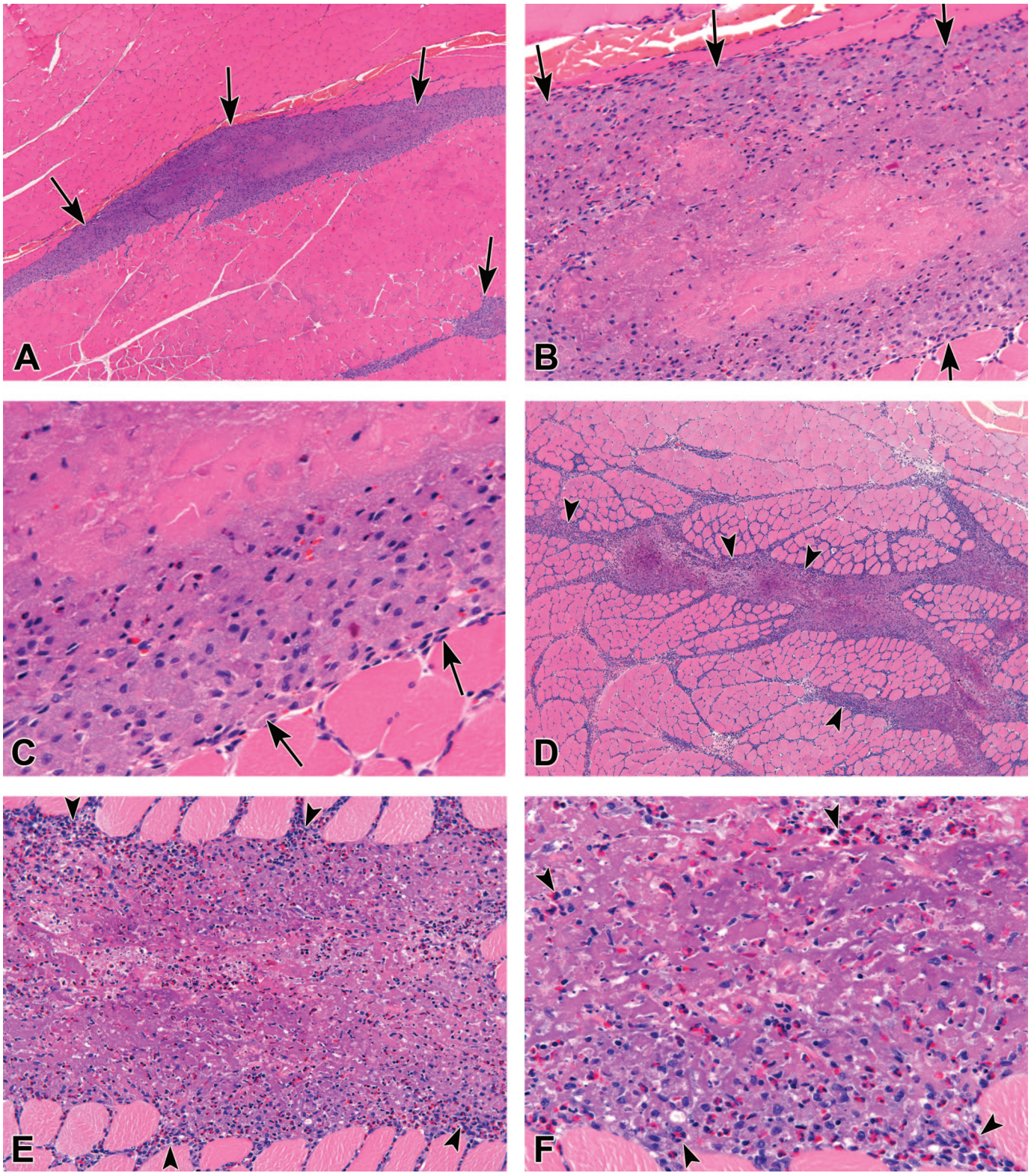
Animal Husbandry and Maintenance

A total of 40 NZW (HsdOkd:NZW) rabbits (20 males and 20 females), at the age of 3 to 4 months at study initiation, were obtained, housed, and treated in Envigo CRS Ltd., Ness-Ziona, Israel Rabbits were housed individually in plastic cages, and were provided a commercial rabbits' diet, approximately 100 g/rabbit/day and allowed free access to drinking water. Acid and chlorine are added to the water, and samples are taken twice a year to ensure they are free of bacteria. Temperature at 17°C to 23°C with a relative humidity of about 30% to 70% was maintained to the maximum extent possible, and their values were verified manually once a day. Twelve-hour light/dark cycle was provided via automatic timer. This study was performed in compliance with the guidelines of the World Health Organization (WHO Technical Report Series, No. 927, 2005, Annex 1—WHO guidelines on nonclinical evaluation of vaccines) and the European Medicines Agency (Guideline on quality, nonclinical, and clinical aspects of live recombinant viral vectored vaccines, EMA/CHMP/VWP/141697/2009, June 2010), and after receiving approval (No. IL-20-12-584) from the Israel National Council for Animal Experimentation. The rabbit has been selected as the species for this study, as it is a well-established and regulatory-accepted animal model for preclinical testing of drugs and vaccines. In addition, the rabbit is a large-enough species enabling IM administration of the required dose volume, and the collection of relatively large volume of arterial blood.

Experimental Design

The experimental design of the study was based on the clinical plan to use 33µg per dose, with two or three IM injections (prime + one or two IM injections).

The animals were randomized to two groups: One group was subjected to four dosing sessions of the test item; C1-RBD (Dyadic International, Inc.) and the second group served as control and was subjected to injections of the same formulation but without C1-RBD (Dyadic International, Inc., Jupiter, FL, USA; Table 1). Vaccine administrations took place at an interval of 1 week between each session during the study. For each dosing session, the test and control items were administered into the right and left quadriceps (thigh) muscles (see also Figure 1). The first IM injections were carried out at the superior area of the right or left muscle, while the second, third, and fourth injections were performed along the muscle to its inferior side. Injections were performed using a 1-ml syringe attached to hypodermic needle. A total volume of 0.5 ml/rabbit/



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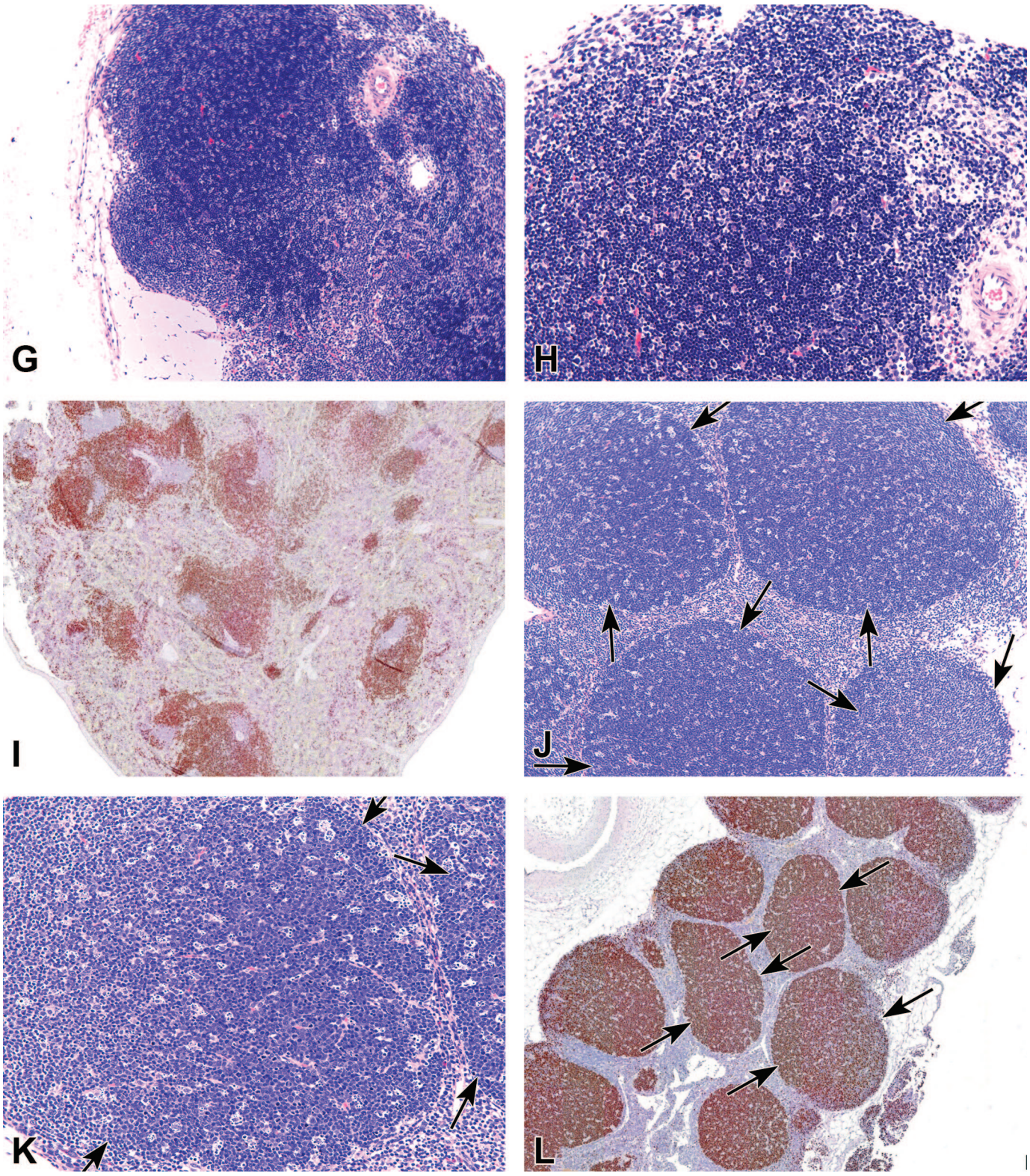

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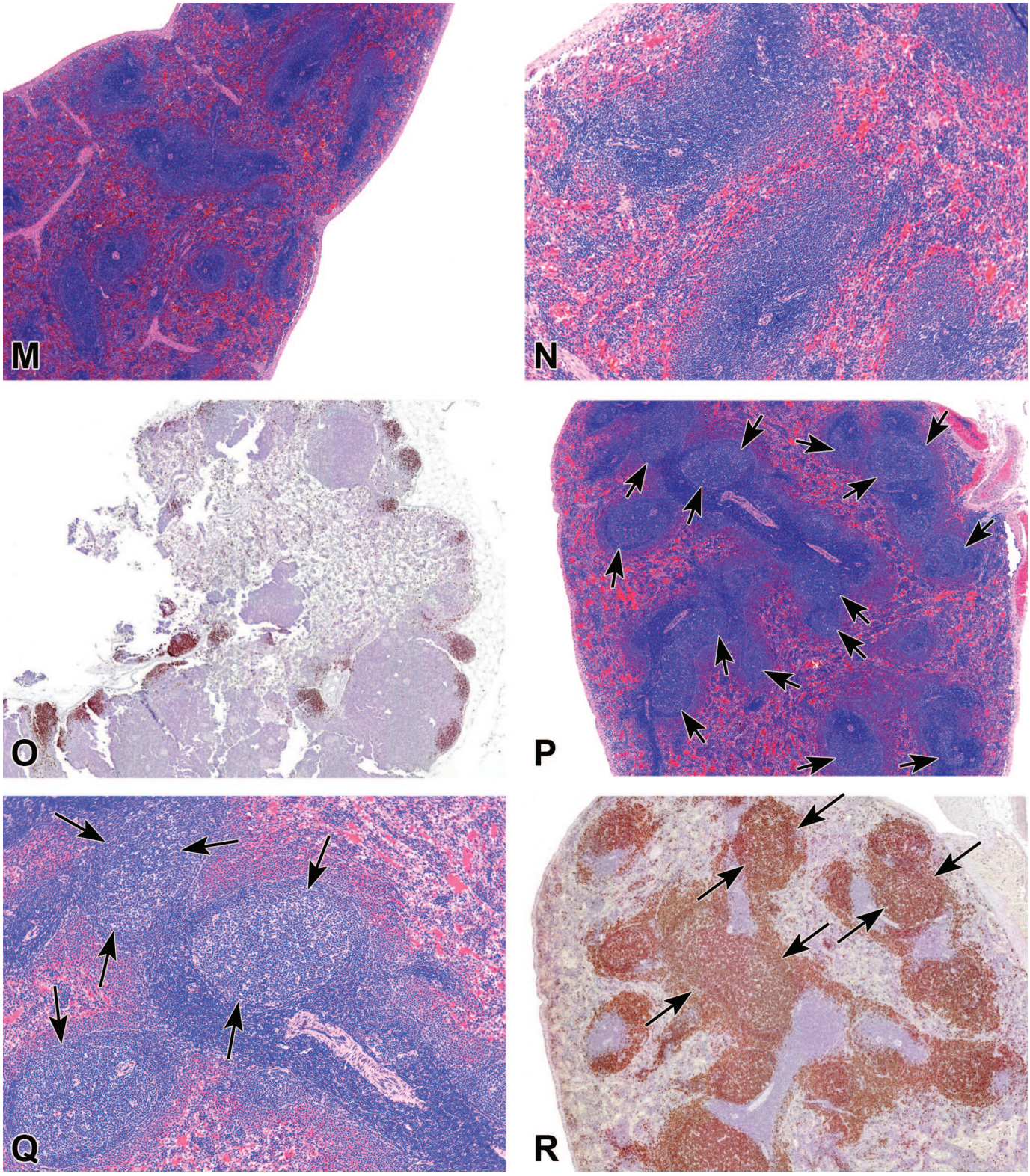

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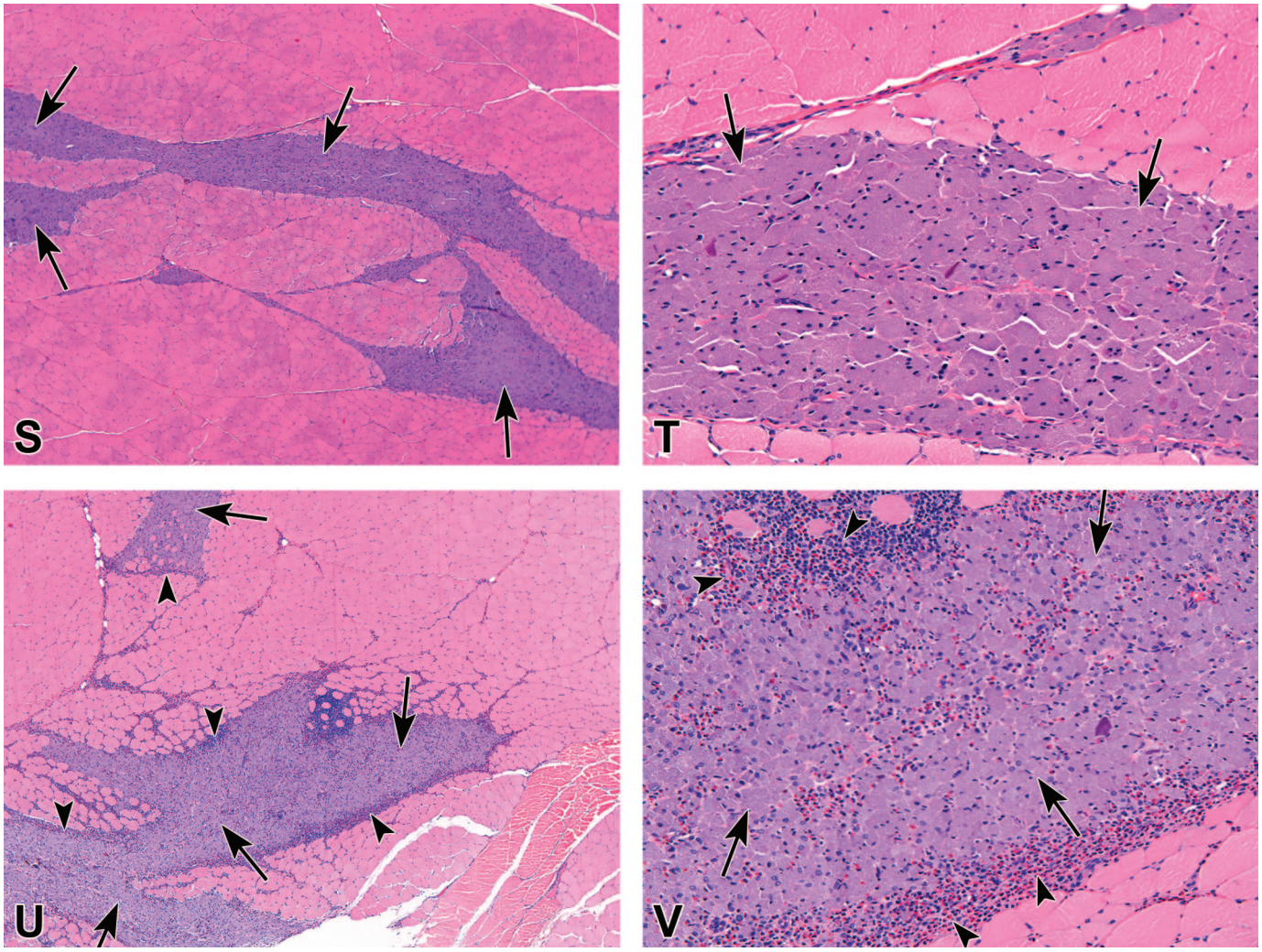

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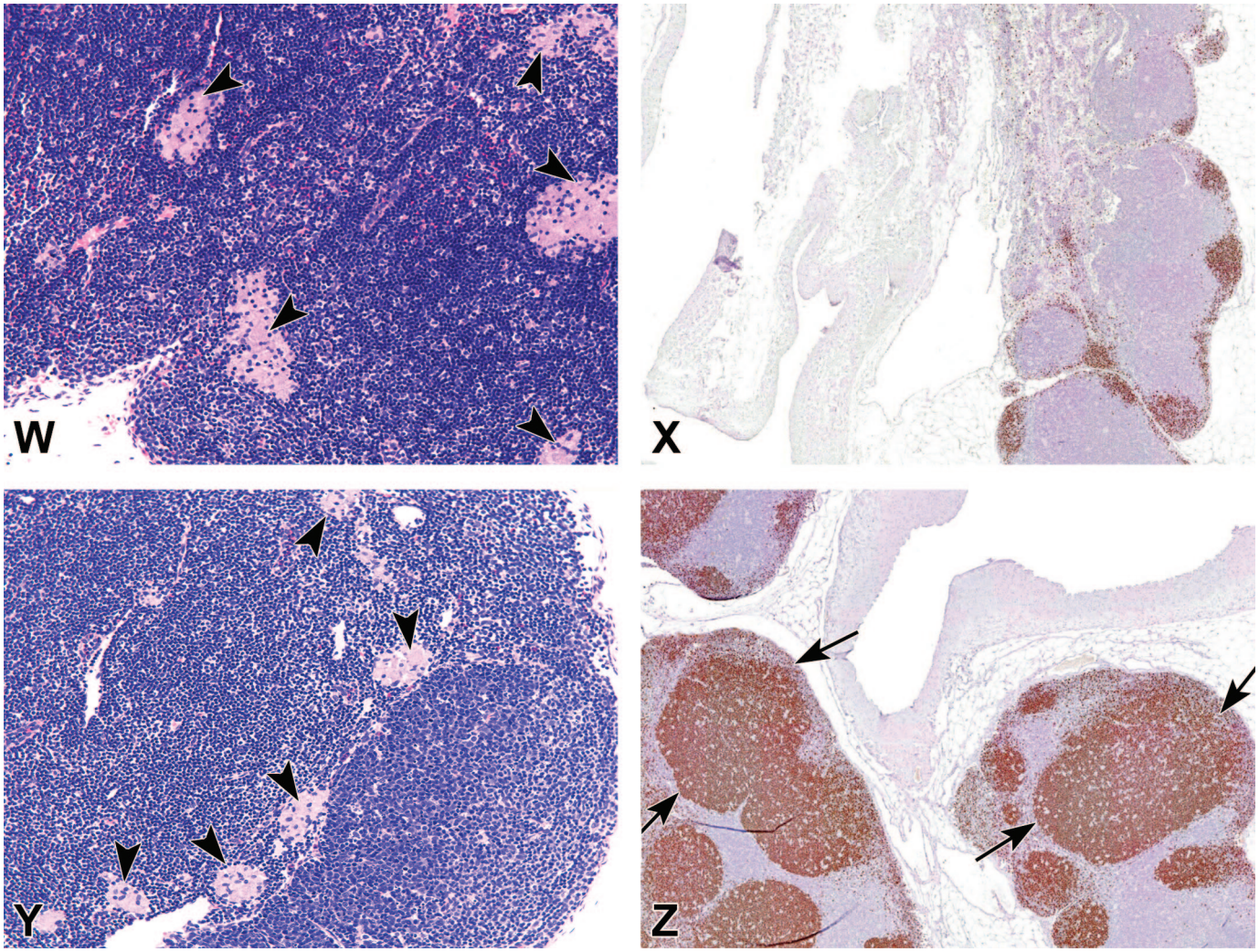

Figure 1. (A-C) Injection site (skeletal muscle) from an animal injected with the placebo (group 1, control item), sacrificed 24 days post first dosing. The lesions consist of mild (grade 2) interstitial infiltration of histiocytes (foreign body granulomatous reaction) associated with degeneration/necrosis of these cells (arrow). No necrosis of the adjacent myofibers was noted. Figure A—original objective x 4; Figure B—original objective x 20; Figure C—original objective x 40. (D-F) Injection site (skeletal muscle) from an animal injected with the C1 SARS-CoV-2 RBD vaccine candidate (group 2, test item), sacrificed 24 days post first dosing. In addition to the mild interstitial infiltration of histiocytes (foreign body granulomatous reaction) associated with degeneration/necrosis of these cells, mild interstitial infiltration of mixed polymorphonuclear cells (heterophils) and mononuclear cells (arrowheads) were noted. No necrosis of the adjacent myofibers was noted. Figure D—original objective x 4; Figure E—original objective x 20; Figure F—original objective x 40. (G, H) The iliac lymph node from an animal injected with the placebo (group 1, control item), sacrificed 24 days post first dosing. Note, no evidence of germinal centers (normal appearance, compared with Figures J and K). Figure G—original objective x 10; Figure H—original objective x 20. (I) The iliac lymph node from an animal injected with the placebo (group 1, control item), sacrificed 24 days post first dosing. Note, no evidence of germinal centers (compared with Figure L). Staining with antibody for PAX-5 (Biocare's PAX clone BC/24, 1:100). (J, K) The iliac lymph node from animal injected with the C1 SARS-CoV-2 RBD vaccine candidate (group 2, test item), sacrificed 24 days post first dosing. The lesions consist of mild germinal centers with increased lymphocytic cellularity (ie, follicular hyperplasia; compared with Figures G and H). Figure J— original objective x 10; Figure K—original objective x 20. (L) The iliac lymph node from an animal injected with the C1 SARS-CoV-2 RBD vaccine candidate (group 2, test item), sacrificed 24 days post first dosing. The lesions consist of mild germinal centers with increased lymphocytic cellularity (ie, follicular hyperplasia). Note the positive staining of the germinal centers (arrows; compared with Figure I). Staining with antibody for PAX-5 (Biocare's PAX clone BC/24, 1:100). (M, N) The spleen from an animal injected with the placebo (group 1, control item), sacrificed 24 days post first dosing. Note, no evidence of germinal centers (normal appearance, compared with Figures P and Q). Figure M—original objective x 10; Figure N—original objective x 20. (O) The spleen from an animal injected with the placebo (group 1, control item), sacrificed 24 days post first dosing. Note, no evidence of germinal centers (compared with Figure R). Staining with antibody for PAX-5 (Biocare's PAX clone BC/24, 1:100). (P, Q) The spleen from an animal injected with the C1 SARS-CoV-2 RBD vaccine candidate (group 2, test item), sacrificed 24 days post first dosing. The lesions consist of mild germinal centers with increased lymphocytic cellularity (ie, follicular hyperplasia; compared with Figures M and N). Figure P—original objective x 10; Figure Q— original objective x 20. (R) The spleen from an animal injected with the C1 SARS-CoV-2 RBD vaccine candidate (group 2, test item), sacrificed 24 days post first dosing. The lesions consist of mild germinal centers with increased lymphocytic cellularity (ie, follicular

Figure 1. (Continued).

hyperplasia). Note the positive staining of the germinal centers (arrows; compared with Figure O). Staining with antibody for PAX-5 (Biocare's PAX clone BC/24, 1:100). (S, T) The injection site (skeletal muscle) from an animal injected with the placebo (group 1, control item), sacrificed 42 days post first dosing (recovery phase). The lesions consist of mild (grade 2) interstitial infiltration of histiocytes (foreign body granulomatous reaction) associated with degeneration/necrosis of these cells (arrows). No necrosis of the adjacent myofibers was noted. Figure S—original objective x 4; Figure T—original objective x 20. (U, V). The injection site (skeletal muscle) from an animal injected with the C1 SARS-CoV-2 RBD vaccine candidate (group 2, test item), sacrificed 42 days post first dosing (recovery phase). The lesions consist, in addition to the mild interstitial infiltration of histiocytic (foreign body granulomatous reaction) associated with degeneration/necrosis of these cells (arrows), also of minimal interstitial infiltration of mixed polymorphonuclear cells (heterophils) and mononuclear cells (arrowheads). No necrosis of the adjacent myofibers was noted (arrows). Figure U—original objective x 4; Figure V—original objective x 20. (W) The iliac lymph node from an animal injected with the placebo (group 1, control item), sacrificed 42 days post first dosing (recovery phase). Note, no evidence of germinal centers (normal appearance, compared with Figure Z). Arrowheads indicate histiocytic cell infiltration. Figure W—x 20. (X) The iliac lymph node from an animal injected with the placebo (group 1, control item), sacrificed 42 days post first dosing. Note, no evidence of germinal centers (compared with Figure Z). Staining with antibody for PAX-5 (Biocare's PAX clone BC/24, 1:100). (Y) The iliac lymph node from an animal injected with the C1 SARS-CoV-2 RBD vaccine candidate (group 2, test item), sacrificed 42 days post first dosing (recovery phase). The lesions consist of mild germinal centers with increased lymphocytic cellularity (ie, follicular hyperplasia). Arrowheads indicate histiocytic cell infiltration (compared with Figure W). Figure Y—original objective x 20. (Z) The iliac lymph node from an animal injected with the C1 SARS-CoV-2 RBD vaccine candidate (group 2, test item), sacrificed 42 days post first dosing. The lesions consist of mild germinal centers with increased lymphocytic cellularity (ie, follicular hyperplasia). Note the positive staining of the germinal centers (arrows; compared with Figure X). Staining with antibody for PAX-5 (Biocare's PAX clone BC/24, 1:100).

dosing session was injected, divided into the right and left injection sites, approximately 0.25 ml/site.

Five male and five female rabbits from each treatment group were sacrificed 24 days after the first injection (3 days following the last dosing session [main phase]). The remaining five male and five female rabbits in each group were sacrificed 42 days after the first injection (3 weeks post the last dosing session [after recovery phase]) to assess the potential reversibility of any adverse reactions or delayed toxicity effects.

Observations and Examinations

All animals were subjected to assessment of systemic clinical signs and local reactions, body weight and body temperature changes (rectal measurements, three hours post dosing and daily for 3 days after dosing), blood sampling for clinical pathology (hematology; biochemistry including creatinine, calcium, glucose, cholesterol, total protein, globulin, aspartate transaminase (AST), lactate dehydrogenase (LDH), potassium, creatine kinase (CPK), high-density lipoprotein (HDL), low density lipoprotein (LDL), phosphorus, urea, albumin, total bilirubin, alanine transaminase (ALT), gamma-glutamyltransferase (GGT), sodium, chloride, triglycerides, and alkaline phosphatase; and coagulation), urine sample analysis (urine samples of approximately 0.4 ml volume were collected from each cage undertray, wrapped by a clean nylon film, following an overnight period, and the following parameters were evaluated using a commercial kit: glucose, ketone, pH, leukocyte, blood, specific gravity, nitrite, bilirubin, urobilinogen, and protein; in addition, urine appearance and color were recorded), and determination of food consumption during the study period. Ophthalmoscopy examination was carried out using an ophthalmoscope after instillation of a mydriatic agent (MYDRAMIDE®), into each eye at least 5 minutes prior to the examination. Both eyes were evaluated for the presence of any abnormalities.

Blood samples for determination of SARS-CoV-2-specific immunoglobulin G (IgG) antibody levels were collected from females assigned to the main phase (only prior to scheduled termination) and from males and females assigned to the recovery phase prior to first dosing, on days 6, 13, and 20 post first dosing and at termination. Blood samples for clinical pathology were collected prior to first dosing, 3 days post first dosing and at termination from the central (auricular) artery (K3EDTA for whole blood, minicollect 9NC coagulation tube for plasma, and minicollect gel tube for serum) and were delivered fresh to laboratory analysis.

At each scheduled necropsy, all animals were subjected to thorough examination, including the external surface of the body; all orifices; cranial, thoracic, and abdominal cavities and their contents. Any abnormalities or gross pathological changes observed in tissues and/or organs were recorded accordingly.

Organs were collected from all animals and fixed in either 10% neutral buffered formalin (approximately 4% formaldehyde solution) or Davidson's Solution (eyes, optic nerves, and harderian glands), for at least 48-hour fixation period. The adrenals, brain, epididymites, heart, kidneys, liver, lungs, ovaries/testes, pituitary, salivary glands, spleen, uterus with cervix, and thymus were weighed wet as soon as possible following their dissection. The thyroid glands were weighed as well but only following fixation of at least 24 hours.

The muscle tissue from each injection site was excised as intact as possible and then two longitudinal horizontal cuts were made in the tissue to provide three muscle samples to facilitate identification of the injection site. Cuts were not made to the full depth of the muscle tissue to preserve orientation. The injection sites (the skin surrounding the thigh muscle) which were marked during the dosing session were also collected.

For the main phase animals, histological examination was performed for the following organs: injection site (leg muscle)

and surrounding skin, brain, spinal cord, pituitary gland, adrenal glands, trachea, esophagus, thyroid, parathyroid, thymus, liver, gallbladder, lungs, heart, aorta, kidneys, urinary bladder, salivary glands, iliac, mesenteric and cervical lymph nodes, spleen, stomach, pancreas, duodenum, jejunum, cecum, tongue, sciatic nerve, skeletal muscle, ileum, colon, rectum, skin, mammary gland, eye, optic nerve, harderian glands, lacrimal glands, testis, epididymis, prostate, seminal vesicles, ovaries, uterus, cervix, vagina, sternum, femur, and knee joint.

To increase the chances to identify the exact area of injection, 3 sections were prepared from each injection site muscle: one in the middle and 2 additional sections at approximately 5 mm proximal and distal to the mid-section. From each section, 3 H&E slides were prepared, total of 9 slides per injected muscle, and 18 slides per animal. In addition, two H&E slides were prepared from the skin covering the injection site of the muscle tissue (total of four slides per animal).

Histopathological examinations from animals assigned to the recovery phase (sacrificed 42 days post last injection) were confined to the following organs/tissues: injection site—muscle (total of nine slides per injected muscle), ileac lymph nodes, mesenteric lymph nodes, and spleen.

Histopathological changes were described and scored using a semiquantitative grading of five grades (0-4):[22] grade 0 = normal (no abnormalities); grade 1 = minimal; grade 2 = mild; grade 3 = moderate; and grade 4 = severe.

PAX-5 Staining of the Spleen and Iliac Lymph Nodes

To highlight the germinal centers, immunohistochemical (IHC) staining was applied on the spleen and iliac lymph nodes of 3 animals/sex/group/24 days timepoint, and on the iliac lymph nodes of 3 animals/sex/group/48 days timepoint. Staining with antibody for PAX-5 diluted 1:100 (Biocare; catalog number CM207B, https://biocare.net/product/pax-5-antibody/), a transcription factor expressed throughout B-cell maturation was used.[23] As a positive control tissue, germinal centers within rabbit lymph node were used.

Immunogenicity

Anti SARS-CoV-2-RBD specific IgG monitoring by direct ELISA (Artemis Bio-Support, Delft). In this assay, SARS-CoV-2 RBD protein was immobilized to the surface of the wells of 96-well µl plates by incubating at 4°C ± 5°C overnight (>16 hours). Unbound SARS-CoV-2 RBD protein was removed by washing with PBS with 0.05% Tween 20 (washing buffer). The rabbit sera were heat-inactivated at 56°C for 30 minutes and diluted at 1:50 in PBS with 0.5% bovine serum albumin (dilution buffer). The standards, positive control rabbit serum, and negative control rabbit serum were also diluted in dilution buffer. The diluted rabbit sera and standards were added to the wells and incubated at 37°C for one hour. After removal of unbound proteins by washing with washing buffer, goat anti-rabbit IgG antibody conjugated with horseradish peroxidase was added and incubated at 21°C ± 5°C for 30 minutes. Following another washing step with washing buffer, the chromogenic substrate 3,3′,5,5′-Tetramethylbenzidine, Liquid Substrate System was added for 10 minutes at 21°C ± 5°C. Finally, the stop solution was added, and the absorbance was measured at 450 nm, using a microplate absorbance reader.

The optical density (OD) value of the blank was subtracted from all OD readings. Using GraphPad Prism5, the OD values of the standards were plotted against their log concentrations. A nonlinear regression line was drawn through these points to construct the standard curve. The concentration of SARS-CoV-2-specific IgG antibodies in each sample was interpolated from this standard curve. Finally, the interpolated concentrations were corrected for the 1:50 dilution.

Statistical Analysis

Calculations were performed using mean SDRelative_01.2.Rnw (validated R-Script for calculations of group mean and standard deviation, Version 2) and Microsoft Excel 365 (paired organ weight means, organ weight to body weight ratio) (both formulas and raw data were quality controlled). Statistical analysis was performed using MultiComp. Rnw (validated R-Script for statistical evaluation between multiple groups and/or multiple parameters between 2 groups). Prior to application of the appropriate statistical method, a normality test was performed considering Gaussian distribution (eg, Shapiro-Wilk normality test; $P < .01$). If the normality test passed for all groups, an equal-variance test was performed (eg, Bartlett test; $P < .01$); if the Bartlett test passed, 1-way analysis of variance with Dunnett's posttest was performed. If the Bartlett test did not pass, Kruskal-Wallis test with Mann-Whitney *U* test was performed. If the normality test did not pass for all groups—Kruskal-Wallis test with Mann-Whitney *U* test was used.

Results

Production and Purification of the DS, C1-RBD Recombinant Subunit Antigen in T. Heterothallica and DP Preparation

The selected C1 production strain was grown in a standard 20-L fermentation as described in the "Methods" section. During development, fermentation yields were continually evaluated by analyzing the secreted proteins. C1-RBD protein was harvested at, or near, peak production after 4 days where production level was approximately 1 g/L. The C1-produced RBD was purified with C-tag affinity chromatography as described in the "Methods" section. The purity of the final C1-RBD antigen was calculated to achieve approximately 97% purity as analyzed by high-performance liquid chromatography (HPLC) reverse-phase affinity chromatography. The purified C1-RBD was formulated with buffered Alhydrogel® '85' 2.0% to contain 66 µg of C1-RBD and 2.5 mg of Alum per 1 ml of the vaccine.

Table 2. Mean severity and incidence of histopathological findings observed at the injection site, inguinal lymph nodes, and spleen.

	Histopathological findings (Number of affected/total number of animals) Scheduled termination (days post first dosing session)							
	24 days				42 days			
	Group 1M	Group 2M	Group 1F	Group 2F	Group 1M	Group 2M	Group 1F	Group 2F
Parameter	($n = 5$)	($n = 5$)	($n = 5$)	($n = 5$)	($n = 5$)	($n = 5$)	($n = 5$)	($n = 5$)
Injection site (Leg muscle) (18 sections—right + left)								
Myofibers—interstitial infiltration of mixed polymorphonuclear cells (heterophils) and mononuclear cells	0.0 (5/5)	2.0 (5/5)	0.0 (5/5)	2.0 (5/5)	0.0 (5/5)	1.0 (5/5)	0.0 (5/5)	1.0 (5/5)
Myofibers—interstitial infiltration of histiocytic cells (foreign body granulomatous reaction) associated with degeneration/ necrosis of these cells	2.0 (5/5)	2.0 (5/5)	2.0 (5/5)	2.0 (5/5)	2.0 (5/5)	2.0 (5/5)	2.0 (5/5)	2.0 (5/5)
Myofibers—mononuclear cell infiltration	0.2 (1/5)	0.0 (5/5)	0.0 (5/5)	0.0 (5/5)	0.0 (5/5)	0.0 (5/5)	0.0 (5/5)	0.0 (5/5)
Injection site (Skin) (2 sections—right + left)	0.0 (5/5)	0.0 (5/5)	0.0 (5/5)	0.0 (5/5)				
Iliac lymph nodes								
Germinal centers with increased lymphocytic cellularity (ie, follicular hyperplasia)	0.0 (5/5)	2.0 (5/5)	0.0 (5/5)	2.0 (5/5)	0.4 (1/5)	2.0 (4/4)	0.0 (5/5)	2.0 (5/5)
Histocytic infiltration	0.0 (5/5)	0.0 (5/5)	0.0 (5/5)	0.0 (5/5)	1.0 (5/5)	1.0 (4/4)	1.0 (5/5)	1.0 (5/5)
Spleen								
Germinal centers with increased lymphocytic cellularity (ie, follicular hyperplasia)	0.0 (5/5)	2.0 (5/5)	0.0 (5/5)	1.6 (5/5)	0.0 (5/5)	0.4 (2/5)	0.2 (1/5)	0.4 (2/5)

Mortality, Clinical Signs, Local Reactions, and Body Weights

No mortality occurred and no abnormal clinical signs were observed in any of the animals throughout the entire observation period.

No edema was evident in any of the injected sites in any of the animals throughout the 42 days observation period, except for one edema in one injection site (designated as grade 1, barely perceptible) in a control male animal which was observed for 3 days (following the first dossing session). Grade of erythema was between grade 0 (no findings) and grade 1 (barely perceptible) and grade 2 (well-defined) on two occasions in one male control animal observed following the first dossing session. No marked difference in erythema scores was noted between the treated group and the control group.

An increase in mean group body weight was evident in all groups throughout the study period and no statistically significant difference was noted in mean group body weight gain at the end of the study period between the treated group and the control group. In both sexes, food consumption was within normal range during acclimation and throughout the study period.

All measurements of body temperature were within the normal body temperature range of rabbits ($\geq$38 and $\leq$39.8, according to the US and European pharmacopeias).

Clinical Pathology

Mean group values of most hematology, biochemistry, and coagulation parameters in vaccine treated group were comparable with that of the control group, within each sex. A few statistically significant differences were found. All these findings were considered incidental since values were within the normal range of reference values and no supporting evidence in clinical signs was observed. In addition, treatment with the C1 SARS-CoV-2 had no effect on hs-C-reactive protein levels compared with the control group.

Urinalysis

There were no marked differences in urinalysis values of the animals assigned to the treated group vs the control group.

Ophthalmoscopy

All groups exhibited normal appearance of blood vessels and the optic disk at the indirect ophthalmoscopy examination, carried out initially during the acclimation period and finally during the last week of the study period.

Organ Weight and Organ Weight to Body Weight Ratio

In the main phase of the female treated group, a statistically significant increase in spleen weight to body weight ratio (*$P <$ .05 vs control) was noted. Additional changes included a statistically significant increase in testis weight to body weight ratio in the recovery phase of the male treated group. In addition, a statistically significant increase in uterus and cervix weight and uterus and cervix weight to body weight ratio and decrease in liver weight to body weight ratio was noted in the recovery phase of the female treated group. These changes were considered incidental since no pathological findings were evident in the histopathological evaluation

Gross Pathological Findings

Gross pathological findings were not observed in any of the animals at the time of their scheduled necropsy excluding a hematoma-like lesion 2 × 1 cm from subcutaneous side at injection site skin in one of the females from control group's main phase.

Histopathological Findings

Main phase (24 days post first dosing). Treatment-related changes were seen in all treated animals. These changes, described below, were seen only at the injection sites, iliac lymph nodes, and spleen, without any lesion suggesting of systemic toxicity (Table 2).

Injection site (skeletal muscle). In the animals of both sexes, injected with the placebo (group 1, control item), the lesions consisted of mild (grade 2) interstitial infiltration of histiocytic cells (foreign body granulomatous reaction) associated with degeneration/necrosis of these cells (Figures A-C). In the animals of both sexes, injected with the C1 SARS-CoV-2 RBD vaccine candidate (group 2, test item), in addition to the interstitial infiltration of histiocytic cells, these foci were associated with mild interstitial infiltration of mixed polymorphonuclear cells (heterophils) and mononuclear cells (Figures D-F). No necrosis of the adjacent myofibers was noted.

Iliac lymph nodes and spleen. Treatment-related lesions were seen only in group 2 of both sexes, consisting of mild germinal centers with increased lymphocytic cellularity (ie, follicular hyperplasia; Figures G, H, J, K, M, N, P, Q).

The results of the IHC staining confirmed the changes seen at histopathology, showing treatment-related increase in size and number of the germinal centers of the iliac lymph nodes and spleen, consisting of B-cell type (Figures I, L, O, R).

Recovery phase (42 days post first dosing). Treatment-related changes were seen in all treated animals. The changes were seen at the same target organs detected in the previous timepoint, that is injection sites, iliac lymph nodes, and spleen, with only a minor trend for recovery at the injection site (Table 2). Even though the mesenteric lymph node was checked in the recovery phase animals, no treatment-related changes were noted in this organ.

Injection site (skeletal muscle). In the animals of both sexes, injected with the placebo (group 1, control item), the lesions consisted of mild (grade 2) interstitial infiltration of histiocytic cells (foreign body granulomatous reaction) associated with degeneration/necrosis of these cells. In the animals of both sexes, injected with the C1 SARS-CoV-2 RBD vaccine candidate (group 2, test item), in addition to the interstitial infiltration of histiocytic cells infiltration, these foci were associated with minimal interstitial infiltration of mixed polymorphonuclear cells (heterophils) and mononuclear cells (in contrast to the mild degree seen at the first sacrifice timepoint (Figures S-V). No necrosis of the adjacent myofibers was noted.

Iliac lymph nodes and spleen. Treatment-related lesions were seen only in group 2 of both sexes, consisting of minimal to mild increased number and size of germinal centers with increased lymphocytic cellularity (ie, follicular hyperplasia). In addition, only in the iliac lymph node of both groups 1 and 2, minimal multifocal histiocytic cell infiltration (aggregation) was noted (Figures W, Y). The nature of the histiocytes was comparable with that seen at the injection site, and therefore was considered as related to the vehicle.

The results of the IHC staining confirmed the changes seen at histopathology, showing treatment-related increase in size and number of the germinal centers of the iliac lymph nodes and spleen, consisting of B-cell type (Figures X, Z).

Immunogenicity

Rabbit serum samples were obtained for rabbit SARS-CoV-2-specific IgG ELISA on days 1, 6, 13, 20, 24, and 42 post vaccination with the C1 SARS-CoV-2 RBD vaccine candidate. None of the serum samples from group 1F and 1M had detectable levels of SARS-CoV-2-specific IgG antibodies, except for serum from one female rabbit. No SARS-CoV-2-specific IgG antibodies were detected in the serum of rabbits from groups 2F and 2M that were obtained on days 1 and 6 post first dosing. However, SARS-CoV-2-specific IgG was detected in the sera of rabbits from group 2F that were obtained on days 13, 20, 24, and 42 post vaccination with the C1 SARS-CoV-2 RBD vaccine candidate, except for one female rabbit, where no SARS-CoV-2-specific IgG was detected on day 13 post first dosing. SARS-CoV-2-specific

IgG was detected in all sera of the rabbits from group 2M that were obtained on days 13, 20, and 42 post first dosing (data not shown). There was a consistent elevation of the antibody concentration throughout the timepoints.

Discussion

This study summarizes the toxicological evaluation of the C1 SARS-CoV-2 RBD vaccine candidate in NZW rabbits, repeatedly administered intramuscularly for a total of four administrations 1 week apart. Treatment-related changes were seen in all treated animals, but only in the injection sites, iliac lymph nodes, and spleen, and no evidence for systemic toxicity was observed.

Indication for the effectiveness of the C1 SARS-CoV-2 RBD vaccine candidate in eliciting an immune response was provided by the formation of follicular hyperplasia in the iliac lymph nodes and in the spleen, in contrast to the vehicle-treated controls, where no changes were seen. In addition, an increase in spleen weight to body weight ratio in the female treated group's main phase was observed, which may also be related to the antigenic stimulation of the test compound.[24] These effects were also mirrored by the ELISA findings, showing the formation of IgG antibodies against RBD only in the C1-RBD-treated animals. The fact that these changes were present throughout the recovery phase as well shows that the reaction provides sustained immunogenic response against RBD.

The inflammatory reaction seen in the injection site of the treated animals was expected. In fact, such inflammatory changes in the injection site of vaccines can be substantial, especially when the vaccine is formulated with an adjuvant.[18,25-27] Furthermore, inflammation at the injection site might even be considered a desired reaction, since it is postulated to increase immune reaction to the injected antigen.[28] When there are no systemic clinical signs and when there is evidence for reversibility, such changes should be considered non-adverse.[26] In our case, the inflammatory changes at the injection sites were mild in nature, and a minor trend toward recovery was observed at the end of the recovery phase. This, in addition to the lack of any systemic signs, further confirms the benign and not adverse nature of this reaction.

In summary, in view of the reported findings and under the conditions of this study, it can be concluded that the C1 SARS-CoV-2 RBD vaccine candidate was not associated with major systemic adverse effects and it is considered safe following four repeated vaccination sessions by IM injections at an interval of 1 week to male and female NZW rabbits. In particular, the fact that germinal centers with increased lymphocytic cellularity (ie, follicular hyperplasia) seen in the regional lymph nodes were sustained throughout the recovery phase as well, in addition to the detection of SARS-CoV-2-specific IgG antibodies in the sera of rabbits on recovery phase, shows that the reaction was long-lasting, and provides the long-lasting immunogenic response against RBD. Further immunogenicity studies with C1 SARS-CoV-2 RBD should be conducted to study how long the immunological activity can be maintained.

Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Ronen Tchelet, Noelia Valbuena Crespo, and Mark Emalfarb work for Dyadic International, Inc., and Hanna Ben-Artzi and Avi Avigdor are employees of BTG—Bio-Technology General (Israel), a Ferring company. These authors have a potential conflict of interest as they may use the vaccine for commercial use.

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