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1 **Title:**

2 Oral subunit SARS-CoV-2 vaccine induces systemic neutralizing IgG, IgA and cellular
3 immune responses and can boost neutralizing antibody responses primed by an injected vaccine

4

5 Jacob Pitcovski[a,c], Nady Gruzdev[a], Anna Abzach[a], Chen Katz[a], Ran Ben-Adiva[a] , Michal
6 Brand Schwartz[a], Itamar Yadid[a,c] , Hadar Haviv[a], Irena Rapoport[a] , Itai Bloch[a], Roy Shadmon[b],
7 Zohar Eitan[b], Dalia Eliahu[a], Talia Hilel[a], Morris Laster[b], Sigal Kremer Tal[b], Tamara Byk
8 Tennenbaum[b], Ehud Shahar[a,c]

9

10 [a]MIGAL Research Institute in the Galilee, Kiryat Shmona , Israel

11 [b]MigVax Ltd.

12 [c]Tel-Hai Academic College , Upper Galilee , Israel.

13 Corresponding author: Ehud Shahar

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15 **Highlights**

16 • MigVax-101 is a multi-epitope oral vaccine for SARS-CoV-2.

17 • MigVax-101 elicits neutralizing IgG and IgA production and cellular responses in mice

18 • MigVax-101 serves as an effective booster in rats to a parenteral anti-S1 vaccine.

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19 **Abstract**

20 The rapid spread of the COVID-19 pandemic, with its devastating medical and economic
21 impacts, triggered an unprecedented race toward development of effective vaccines. The
22 commercialized vaccines are parenterally administered, which poses logistic challenges, while
23 adequate protection at the mucosal sites of virus entry is questionable. Furthermore, essentially
24 all vaccine candidates target the viral spike (S) protein, a surface protein that undergoes
25 significant antigenic drift. This work aimed to develop an oral multi-antigen SARS-CoV-2
26 vaccine comprised of the receptor binding domain (RBD) of the viral S protein, two domains of
27 the viral nucleocapsid protein (N), and heat-labile enterotoxin B (LTB), a potent mucosal
28 adjuvant. The humoral, mucosal and cell-mediated immune responses of both a three-dose
29 vaccination schedule and a heterologous subcutaneous prime and oral booster regimen were
30 assessed in mice and rats, respectively. Mice receiving the oral vaccine compared to control
31 mice showed significantly enhanced post-dose-3 virus-neutralizing antibody, anti-S IgG and IgA
32 production and N-protein-stimulated IFN-γ and IL-2 secretion by T cells. When administered as
33 a booster to rats following parenteral priming with the viral S1 protein, the oral vaccine elicited
34 markedly higher neutralizing antibody titres than did oral placebo booster. A single oral booster
35 following two subcutaneous priming doses elicited serum IgG and mucosal IgA levels similar to
36 those raised by three subcutaneous doses. In conclusion, the oral LTB-adjuvanted multi-epitope
37 SARS-CoV-2 vaccine triggered versatile humoral, cellular and mucosal immune responses,
38 which are likely to provide protection, while also minimizing technical hurdles presently limiting
39 global vaccination, whether by priming or booster programs.

40 **Keywords**

41 SARS-CoV-2, oral vaccine, subunit vaccine, heterologous boost, nucleocapsid, Spike-
42 RBD.

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43 **INTRODUCTION**

44 The rapid spread of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-
45 mediated coronavirus disease 2019 (COVID-19) pandemic, its related mortality and morbidity
46 rates [1], and heavy toll on healthcare and economic systems across the globe have triggered
47 unprecedented effort to develop and mass-produce safe and effective vaccines. Over 100
48 candidate vaccines in various stages of clinical development and over 180 in preclinical
49 development including those based on mRNA, non-replicating viral vectors, recombinant
50 proteins, inactivated virus, and DNA vaccines [2], almost all of which target S protein.
51 Limitations of some of these vaccination strategies include the possibility of a live vaccine
52 reverting to the virulent state in immunocompromised hosts, as well as potential adverse effects,
53 including allergic and autoimmune reactions. In addition, protein antigen-based vaccines have
54 been a very successful platform for many licensed vaccines, thus are widely studied in vaccine
55 development [3].

56 While intramuscular and subcutaneously delivered vaccines elicit systemic immune responses,
57 they generally fail to induce mucosal immunity, which provides the first barrier against
58 pathogens infiltrating at the mucosal surface. Among mucosal routes, oral vaccines are
59 logistically less challenging by avoiding the need for needles, may be associated with superior
60 patient compliance among needle-phobic subjects compared to injected vaccines, and offer the
61 opportunity for self-administration. These issues could contribute to potentially improved
62 success of mass vaccination, particularly during pandemics. Extensive efforts have been invested
63 into developing protein-based mucosal vaccines for infectious diseases such as Dengue [4],
64 influenza [5], tetanus [6], diphtheria [7], hepatitis [8], and MERS-CoV [9]. There are no
65 approved human oral or intranasal protein-based vaccines, given that oral vaccines generally
66 suffer from low stability and suboptimal induction of concerted antibody and cellular immune
67 responses.

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68 To overcome some of these limitations, live bacterial cells or bacterial components have been
69 proposed as carriers of recombinant antigens, due to their potent immunostimulating effect. One
70 such polypeptide, LTB, is the non-toxic B subunit of *E. coli* heat-labile enterotoxin (LT), an
71 established potent mucosal immunogen, which has been broadly applied in several vaccine
72 development studies, both as a free adjuvant and in chemical conjugation or genetic fusion with
73 various antigens [10-15]. For example, mixing of purified LTB to recombinant knob protein of
74 egg drop syndrome adenovirus significantly augmented antibody responses in orally and
75 transcutaneously vaccinated chickens [16]. LTB adjuvant properties have also been shown upon
76 oral co-administration of HPV16L1 with LTB, which induced higher IgG and IgA titres as
77 compared to non-adjuvanted controls [17]. Rios-Huerta et al. [18] reported on significant
78 production of secretory IgA by BALB/c mice orally immunized with tobacco leaf tissue extracts
79 containing a chimeric LTB-EBOV protein bearing two *Zaire ebolavirus* GP1 protein epitopes. A
80 recombinant subunit vaccine (rLTBR1) comprised of the R repeat region of P97 adhesin of *M.*
81 *hyopneumoniae* (R1) fused to LTB, elicited high levels of systemic and mucosal antibodies in
82 BALB/c mice inoculated by the intranasal or intramuscular routes [19]. Another study showed
83 that systemic anti-R1 antibody levels were significantly higher in mice orally vaccinated with
84 recombinant R1-LTB protein compared to those vaccinated with R1 alone. In line with these
85 reports, LTB fusion with the C-terminal fragments of botulinum neurotoxins (BoNTs) serotypes
86 C and D [20], *hyopneumoniae* antigens [21], *A. pleuropneumoniae* toxin epitopes [22], dengue
87 envelope protein domain III-LTB [4], porcine epidemic diarrhoea virus spike protein [23] and
88 influenza A virus epitopes (IAVe) [24], induced broad humoral and cellular immune responses
89 and improved protection against viral challenge in various animal models.

90 The CoV genome of the enveloped, positive-stranded RNA SARS-CoV-2 encodes non-structural
91 replicases, as well as the spike (S), envelope (E), membrane (M) and nucleocapsid (N) structural
92 proteins [25]. S protein is comprised of S1 and S2 domains, with S1 bearing a receptor-binding
93 domain (RBD), which binds host angiotensin-converting enzyme (ACE2) [26, 27], for viral entry

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94 into cells. While the S protein is the central focus of currently available SARS-CoV-2 vaccines,
95 its rapid evolution, enabling viral evasion of host immune responses, has raised concerns
96 regarding the breadth of protection it can provide against circulating mutant strains [28].
97 Dominance of T cells targeting viral components other than S has been identified in the serum of
98 convalescent COVID-19 patients [29-31], suggesting the importance of expanding the epitope
99 repertoire of vaccines under development.

100

101 The present work aimed to develop an oral, multi-antigen SARS-CoV-2 vaccine comprised of
102 either the receptor binding domain (RBD) or S1 domain of the viral Spike (S) glycoprotein, two
103 domains of the viral N protein, each fused to LTB, and free LTB. The humoral, mucosal and
104 cell-mediated immune responses of both a homologous oral vaccination schedule (using only
105 oral vaccine for all doses) and a heterologous subcutaneous prime and oral booster regimen were
106 assessed in mice and rats, respectively. The ability of an oral vaccine to function as a booster to
107 subjects immunized with other vaccines, all of which are given systemically, is especially
108 pertinent for the control of COVID-19 given that an increasing number of people are being
109 immunized and will have future repeated needs for booster doses as is the case for other
110 vaccines.

111

112 MATERIALS AND METHODS

113

114 Protein production

115 *Plasmid construction*

116 Synthetic constructs encoding LTB and chimeric LTB-NC (-terminal domain of nucleocapsid
117 protein), linked by a 6 aa linker and including a C-terminal HIS-tag were prepared by

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118 Genscript® (Piscataway, NJ). A synthetic linear construct encoding LTB-NN (n-terminal
119 domain of N protein), linked by a 6 aa linker and including a C-terminal HIS-tag was prepared
120 by IDT-DNA (Coralville, IA). Constructs were PCR-amplified (Table 1). For cloning into
121 pET28a, NcoI and XhoI sequences were incorporated into the forward and reverse primers,
122 respectively.

123

124 Table 1. Primers used for cloning LTB and LTB chimeric proteins.

Gene	Primer	Sequence*
LTB-NC	For	CATG**CCATGG**GCGCGGCGGAGGCG
	Rev	CCG**CTCGAG**GGTTTTATACGCGTCAATGTGC
LTB-NN	For	CATG**CCATGG**GCAACAACACCGCGAGC
	Rev	CCG**CTCGAG**GCTGCCTTCCGCGTAAAACC
LTB	For	CATG**CCATGG**GCAACAAGGTGAAATGCTACG
	Rev	CCG**CTCGAG**GTTTTTCATGCTAATCGCCGCGATGC

125 *Bold underlined nucleotides indicate restriction sites.

126

127 *Transformation and protein expression*

128 Plasmids were electro-transformed into *E. coli* C41 (Lucigen) and plated on LB-agar
129 supplemented with 50µg/mL kanamycin and 1% glucose. Colonies carrying the plasmid were
130 grown in a shaker incubator in LB medium (10 g/l bactotryptone, 5g/L yeast extract)
131 supplemented with 1% glucose, 100 µg/mL kanamycin, at starters/flask volume ratio: 1/100 and
132 then in 2×YT medium (16 g/l bactotryptone, 10 g/l yeast extract, 5 g/L NaCl), containing 100
133 µg/mL kanamycin, at 37 °C at 250 rpm, A_{600} reached 0.6. The growth temperature was lowered

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134 to 25°C, and after 20 min, 0.4 mM IPTG was added. Cells were further grown for 16 hours at
135 25°C, collected (4,500 x g, 20 min, 4 °C) and then lysed by sonication (45 A, 5 sec on/10 sec off,
136 3 repeats with 10 min rest between intervals, on ice) with lysis buffer (50 mM phosphate buffer
137 pH 7.2, 150 mM NaCl, 0.1% Tween 20 (latter for LTB-NC and LTB-NN) 1 tablet of protease
138 inhibitor per 500 ml culture)). The lysate was clarified by centrifugation (2 cycles of 15,000 x g,
139 20 min, 4 °C).

140 *Protein purification*

141 Protein was purified on an Econo-Pack® BIO-RAD gravity column with 0.5 mL immobilized D-
142 galactose-agarose resin (Pierce, Thermo) (50% slurry), equilibrated with binding buffer (50 mM
143 phosphate buffer pH 7.2, 150 mM NaCl). The clarified lysate was loaded on the column, which
144 was then washed with binding buffer. Protein was eluted using elution buffer (50 mM phosphate
145 buffer pH 7.2, 150 mM NaCl, 100 mM galactose). Fractions were analysed by 12% SDS-PAGE
146 and immunoblot, and relevant fractions were pooled. LTB and LTB-NC samples were dialyzed
147 three times (1:100, 3.5KDa) against 50 mM phosphate buffer pH 7.2, 150 mM NaCl, after which
148 15% (v/v) glycerol was added. For LTB-NN samples, 0.5% Tween 20 was added and samples
149 were dialyzed against 50 mM phosphate buffer pH 7.2, 150 mM NaCl +0.1% Tween 20.

150 Following dialysis, protein concentration was quantified using Nanodrop (Thermo Fisher
151 Scientific, Waltham, MA) and Bradford (BioRad, Hercules, CA). LTB was quantified using an
152 enzyme-linked immunosorbent assay (ELISA), with a calibration curve prepared as previous
153 described [32]. Briefly, plates were coated with GM1, and after blocking, eluted proteins were
154 incubated in the plates. Detection was performed using rabbit anti-cholera toxin IgG, followed
155 by incubation with goat anti-rabbit peroxidase IgG. If needed, Amicon Ultra-15 (Merck) was
156 used to concentrate the protein to 1-2 mg/mL before storage. All proteins were aliquoted and
157 stored at -80°C.

158

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159 **Animal immunization**

160 All animal studies were approved by the institutional Committee for Ethical Conduct in the Care
161 and Use of Laboratory Animals and abided by guidelines set forth by the Animal Welfare Law
162 (Animal Studies) - 1994 (State of Israel), Guide for the Care and Use of Laboratory Animals, the
163 Institute of Laboratory Animal Research (ILAR); Guidelines of the National Institute of Health
164 (NIH), and Association for Assessment and Accreditation of Laboratory Animal Care
165 (AAALAC).

166 *Homologous oral vaccination of mice*

167 The mouse vaccination experiment was performed by "Science in Action", Ness Ziona, Israel.
168 Ten BALBc (5 males and 5 females), 8-week-old mice per treatment group were inoculated
169 orally or by gastric gavage on days 0, 14, and 28. Mice receiving oral vaccine were administered
170 a combination of S1 (GenScript), LTB-NN, LTB-NC and free LTB, at either a high dose (HD)
171 (88 µg, 9 µg, 35 µg and 20 µg, respectively) or low dose (LD) (18 µg, 9 µg, 7 µg and 4 µg,
172 respectively) or HD vaccine without the free LTB component (herein oHD-LTB, oLD-LTB and
173 oHD, respectively). Mice administered the vaccine by gastric gavage received the high dose with
174 free LTB (gHD-LTB). Control mice were treated with an oral dose of PBS. Blood samples were
175 drawn on days 26 and 49. After the mice were sacrificed, wet faeces samples were collected
176 from colon and spleens were harvested.

177 Blood samples were allowed to clot for 30 min, then centrifuged (3000 x g, 10 min, 22 °C), and
178 serum was collected and stored at -70 °C until analysis. Faeces samples were frozen at -20 °C
179 until further use.

180

181 *Rat 'heterologous' systemic-prime oral-boost immunization*

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182 This study was performed by Vivox, Nesher, Israel. Nine (4 males and 5 females) 8-weeks-old
183 Sprague Dawley rats per treatment group, by subcutaneous injection with 50µL S1 (Genscript)
184 (50µg/rat) administered once (mixed 1:1 (v/v) with Freund's complete adjuvant (Sigma
185 Aldrich)) or twice (second dose mixed with Freund's incomplete adjuvant), at a 14-day interval.
186 Two weeks after the last priming dose, oral MigVax-101 (RBD 90 µg (Baylor College of
187 Medicine, Houston, Texas) + LTB 35 µg + LTB-NN 70 µg +LTB-NC 70 µg) was administered
188 once or twice, with a 14-day interval between the doses (Fig. 1). Control rats were injected
189 subcutaneously with one or two priming doses of S1, followed by one or two oral doses of DP
190 buffer (50 mM phosphate buffer, pH=7.2, 150 mM NaCl, 0.1% Tween 20, 15% glycerol) or
191 booster vaccination with a third subcutaneous dose of S1 (mixed with Freund's incomplete
192 adjuvant). Oral doses were administered by gently dripping the solution on the top of the tongue,
193 just beyond the lip line, using a syringe attached to a gavage cannula. Animals were sacrificed 14
194 days after administration of the last booster dose.

195 Venous blood was collected from the retro-orbital sinus prior to immunization and 14 days after
196 administration of the last booster dose. Serum was prepared as described above. Bronchoalveolar
197 lavage fluid (BALF) was obtained from harvested lungs by slowly washing the lungs with 1.5
198 mL PBS, after which, the fluid was collected and mixed with 0.5 mL PBS. The solution was
199 then used to wash the lungs twice. Collected fluid was then centrifuged (400 x g, 5 min, 22 °C)
200 and frozen at -70 °C until analysis.

201 **Evaluation of oral vaccine immunogenicity**

202 To prepare for analysis, faeces samples were thawed on ice and weighed. Extraction buffer
203 (PBS+ 0.05% Tween 20 (PBST) + 5% skim milk + 1mM PMSF) was then added (1:1 v/w), and
204 samples were vortexed until uniformity. Samples were then centrifuged at 16,000g, 4°C for 15
205 min, after which, the supernatant was collected and stored at -20 °C until analysis. Spleens were
206 kept cooled on ice prior to processing. Spleens were dissociated in 5 mL PBS using a

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207 gentleMACS™ dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Following
208 centrifugation (800xg, 5 min, at room temperature), pellets were collected and erythrocytes were
209 lysed with distilled water, splenocytes were passed through a 70-µm cell strainer (BD
210 Biosciences, Bedford, MA), then washed with PBS and transferred to cell culture medium
211 (RPMI 1640 supplemented with 1% foetal bovine serum (FBS) and 1% penicillin streptomycin
212 solution (Biological Industries, Beit HaEmek, Israel). Mice cells for theexperiment, were
213 analysed fresh.

214

215 **Assessment of humoral immune response**

216 *Determination of anti S1 IgG and IgA levels*

217 ELISA was performed to determine anti-S1 IgG and IgA levels. Plates were coated overnight
218 with 100 ng/well of S1 (Genscript) in coating buffer (0.015M carbonate/bicarbonate buffer, pH =
219 9.6) (4 °C) and then blocked with blocking buffer (5% skim milk in PBST) (1 hr, room
220 temperature). To determine IgG titres, sera samples were serially diluted in blocking buffer, then
221 added to each well and incubated for 1 hr at 37 °C. Thereafter, plates were rinsed three times
222 with PBST, and then incubated with peroxidase-conjugated goat anti-mouse IgG or goat anti-rat
223 IgG (Abcam, Cambridge, UK) for 1 hr, at 37 °C. To determine IgA levels, undiluted BALF or
224 processed faeces samples were added to plates and incubated for 1 h at 37 °C. Thereafter, plates
225 were rinsed three times with PBST and incubated with peroxidase-conjugated goat anti-mouse
226 IgA or goat anti-rat IgA (Abcam), for 1 hr, at 37 °C. Plates were then rinsed with PBST and
227 incubated with 3,3′,5,5′-tetramethylbenzidine (TMB, Southern Biotech, Birmingham, AL, USA).
228 Absorbance was measured at 650 nm using an Infinite M200 pro plate reader (Tecan,
229 Männedorf, Switzerland). IgA values were normalized by dividing absorbance of specific anti-
230 S1 IgA by the total IgA level measured in the sample.

231 *Serum neutralization assays*

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232 The cPass neutralization assay was performed with mouse sera, according to the manufacturer's
233 instructions (Genscript). A SARS-CoV-2 pseudo-virus neutralization assay was performed with
234 rat sera at the Israeli Central Virology Lab (Sheba Medical Center, Tel Hashomer, Israel). The
235 propagation-competent vesicular stomatitis virus expressing cSARS-CoV-2 S protein and
236 carrying the gene encoding green fluorescence protein (psSARS-2) was used in an assay similar
237 to a recently reported assay [33] shown to correlate well withauthentic SARS-CoV-2 virus
238 micro-neutralization assay. Following titration, 100 focus forming units (ffu) of psSARS-2 were
239 incubated with 2-fold serial dilutions of heat-inactivated (56°C, 30 min) immune rat sera. After
240 incubation for 60 min at 37°C, the virus/serum mixtures were transferred to Vero E6 cells that
241 had been grown to confluence in 96-well plates, and incubated for 90 min at 37°C. Thereafter,
242 1% methyl cellulose in Dulbecco's modified eagle's medium (DMEM) with 2% fetal bovine
243 serum (FBS) was added, and plates were incubated for 24h, after which, 50% plaque reduction
244 titre was calculated by counting green fluorescent foci using a fluorescence microscope (EVOS
245 M5000, Invitrogen).

246

247 **Assessment of cell-mediated immunity**

248 *Splenocyte induction*

249 Splenocytes (3.75×10^6 cells in 750 µl cell culture medium) from each mouse were seeded in
250 24-well plates and allowed to settle for 1 hr, before being mixed 1:1 with medium (RPMI 1640
251 supplemented with 1% FBS and 1% penicillin streptomycin solution (Biological Industries)
252 containing 20 µg/mL N (Sino Biological, Beijing, China) or S1 proteins. Phorbol-myristate
253 acetate (PMA) (5 ng/mL)-ionomycin (1 µg/mL) and culture medium were added to positive and
254 negative controls, respectively. Plates were then incubated overnight, at 37 °C, with 5% CO_2.
255 Thereafter, samples (100 µL) were transferred to black optic-bottom 96-well plates and to
256 ELISPOT plates (CTL, Bonn, Germany) to assay cell proliferation and number of IFN-γ-

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257 secreting T cells, respectively, and incubated for 24 hr, at 37 °C with 5% CO_2. Proliferation was
258 measured after incubation of cells (4 hr, 37 °C) with 10 µL ALAMAR blue. Fluorescence was
259 measured (560 nm/590 nm), and blank readings were subtracted from readings of all wells
260 containing proteins. To quantify IFN-γ-secreting T cells, plates were washed, and then stained
261 with anti-IFN–γ-peroxidase antibodies and substrate, as per the manufacturer's instructions.
262 Spots were counted with an ELISpot reader (CTL). The number of spots obtained from cells
263 incubated without the stimulating protein was subtracted from the number obtained from cells
264 incubated with the protein.

265 Levels of IL-2 and IFN-γ (Th1 immune markers) and IL-4 and IL-10 (Th2 immune
266 markers)cytokines secreted to the supernatant were determined using specific ELISA kits
267 according to the manufacturer's instructions (Peprotech, Rehovot, Israel).

268 **Safety assessment of oral vaccine**

269 Vaccine safety was determined by Envigo, Ness Ziona, Israel. Sprague Dawley rats (10-14 per
270 treatment group, 8-weeks-old, equal number of male and females) were orally immunized 2 or 3
271 times at 14-day intervals with 100 µL MigVax-101 or with DP buffer as negative control.
272 Weight and body core temperature were monitored throughout the study period. Animals were
273 sacrificed 2 days after the second dose (day 16) or third dose (day 30) or 3 weeks after the third
274 dose (day 49). On the day they were sacrificed, blood was drawn and subjected to standard
275 haematology, biochemistry and coagulation testing. Brain, cervical lymph nodes, tongue,
276 oesophagus, heart, mediastinal lymph nodes, lungs, thymus, spleen, kidneys, stomach,
277 duodenum, liver, femur (bone marrow) and skull (buccal mucosa) tissues were harvested, fixed
278 in formalin, and histopathologically assessed for toxicological signs.

279 **Statistical analysis**

280 Data are presented as mean ± standard deviation. Statistical significance was assessed using the
281 paired or unpaired, one-tailed student's T test, analysis of variance (ANOVA) Tukey or ANOVA

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282 Dunnett test, as indicated in figure legends. All statistical analyses were performed using Prism
283 (GraphPad).
284

285 **RESULTS**

286 **Increased anti-S1 IgG and IgA and antibody neutralizing levels following homologous oral**
287 **vaccination**

288 Mice antibody responses were quantified 26 and 49 days post-first immunization (dpi), which
289 corresponded with 12 days after the second and 21 days after the third vaccination. Significant
290 elevations in anti-S1 IgG levels were measured in the sera of all mice receiving oral
291 vaccinations, as compared to control mice (2.2-2.7-fold, $P<0.01$) and mice receiving gavage
292 vaccination (gHD-LTB; 1.9-2.4-fold, $P<0.01$) (Fig. 2a). On 49 dpi, mice administered oHD-LTB
293 exhibited anti-S1 IgG levels ($p=0.0253$) that were significantly higher than those measured on 26
294 dpi, whereas levels in the other two oral vaccination groups showed smaller increments in IgG
295 levels (Fig. 2b). No change in IgG levels was noted after the third gavage vaccination (Fig. 2b).
296 Three oral vaccinations with oHD and oHD-LTB induced a significant rise in secretory anti-S1
297 IgA levels as compared to the negative PBS-treated control (Fig. 2c, 14.6-fold, $p<0.001$ and 9.3-
298 fold, $p<0.05$, respectively).

299 Mice immunization with oHD-LTB and oHD provided for significantly higher neutralization
300 than both the gHD-LTB and the control groups (Fig. 3).

301 Cellular immune responses

302 Significant increases in the proliferation of splenocytes collected from mice vaccinated with
303 oHD-LTB ($p<0.01$) or oHD ($p<0.05$) as compared to those collected from PBS-treated mice,
304 were observed following N induction (Fig. 4a). In addition, IFN-γ-secreting T-cell counts were
305 significantly higher among splenocytes from oHD-LTB mice as compared to splenocytes from

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306 all other test groups (Fig. 4b, p<0.001). Following splenocyte stimulation with N protein, IL-2
307 levels secreted by cells collected from oHD-LTB mice were significantly higher (p<0.01 or
308 0.001) than those secreted by splenocytes of all other treatment groups (Fig. 4c). No significant
309 intergroup differences were noted with regard to secreted levels of IFNγ, or the Th-2 related
310 cytokines IL-10 and IL-4. Following S1 stimulation, splenocytes derived from oHD-LTB and
311 oLD-LTB mice secreted significantly higher IFNγ levels as compared to the gHD-LTB
312 splenocytes (p<0.05) (Fig. 4d).

313 **Oral boosting after S1 injection increases production of neutralizing antibodies**

314 Having established that the oral vaccine is immunogenic in mice, we wished to evaluate the
315 ability of the oral vaccine to act as a booster vaccine in rats that had been immunized with a
316 model systemic vaccine (Fig. 1) Antibody titres were not further elevated following a second
317 oral booster (Fig. 5a). Administration of a MigVax-101 oral boost after one or two injections of
318 S1 did not significantly increase anti-S1 IgG as compared to placebo (Fig. 5a). Anti-S1 IgA
319 levels measured in BALF were increased after priming with one S1 injection and boosting with
320 one oral MigVax-101 dose as compared to rats receiving a single oral placebo boost (Fig. 5b).
321 Furthermore, two subcutaneous S1 doses, followed by one oral MigVax-101 boost elicited IgA
322 levels at least as high as those obtained following three injections (Fig. 5b). Similarly, two oral
323 MigVax-101 doses after two injections were associated with non-significant increase in anti-S1
324 IgA levels as compared to two injections followed by two oral placebo administrations (Fig. 5b).

325 The levels of neutralizing antibodies were elevated in the serum of rats vaccinated with injected
326 S1 protein following a heterologous oral boost with MigVax-101. For all vaccination
327 schedules, rats receiving the oral booster showed significantly higher neutralizing antibody titres
328 than those treated with an oral placebo booster (Fig. 6). A double subcutaneous priming regimen,
329 followed by a single oral MigVax-101 booster, or a third subcutaneous S1 injection booster,
330 yielded similar neutralizing antibody titres 14 days after the boost in the oral and injectable

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331 boosts groups. Both were significantly higher than two injections alone followed by placebo at
332 this time point. (Fig. 6).

333 **Safety**

334 Safety testing was performed on rats given the high dose MigVax-101 concentrations with 14
335 days interval between immunizations. Comprehensive toxicology examinations were performed
336 to rule-out short term, 2 days post 2nd and 3rd dose, and long term, 3 weeks post 3rd doses,
337 adverse responses. Blood testing and organ histopathology found no significant toxicological
338 effects in animals subjected to any of the tested vaccine regimens.

339

340 **Discussion**

341 The current study showed the safety and immunogenicity in mice of a three-dose vaccination
342 regimen with an oral multi-epitope SARS-CoV-2 vaccine, as manifested by increased levels of
343 S1-specific IgG, IgA, and virus-neutralizing antibodies. The benefit of the inclusion of LTB, a
344 potent mucosal adjuvant, in the vaccine formulation was evidenced by elevated anti-S1 IgG
345 levels in oHD-LTB-vaccinated, oLD-LTB-vaccinated and oHD-vaccinated mice, all of which
346 also elicited titres comparable to those measured in sera of convalescent COVID-19 patients
347 [34]. Notably, animal IgG responses rose with repeat oral vaccine dosing, while gavage
348 vaccination responses plateaued after two doses and were generally low. In this study, gavage
349 data underscores LTB's role in antigen presentation in the oropharyngeal cavity. Once being
350 bypassed, immune response was similar to the negative control. As stomach pH is acidic and
351 contains digestive enzymes, vaccine proteins could have been denatured and not reach the small
352 intestine mucosa to evoke substantial mucosal immunity.

353 In another aspect, data variability may have been, at least partially, attributed to ununiformed
354 time of exposure of the administered vaccine at the rodent's oral cavity.

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355

356 oHD-LTB was associated with more intense Th1 responses to S or N antigens, as shown by
357 higher IFN-γ-secreting T-cell counts and cytokine secretion, as compared to other test groups.
358 In addition, No shifts in the CD8 to CD4 population ratio were noted in any treatment group (as
359 determined by FACS; data not shown).

360 When administered as a booster to rats that had been subcutaneously immunized with viral S1
361 protein, MigVax-101 markedly enhanced neutralizing antibody levels, with the effect of a single
362 oral booster following two injected S1 doses corresponding to that following three injected
363 doses. While the oral boosters did not increase IgG titres, they enhanced mucosal antibody
364 responses as compared to two or three S1 injections. These findings are consistent with those
365 reported by Tan et al. [35], who compared the performance of recombinant S and RBD proteins,
366 formulated with an adjuvant or monophosphoryl lipid A liposomes, in both homologous and
367 heterologous prime-boost intramuscular vaccination regimens. They found that compared to S,
368 RBD induced low primary immunity in rodents, but was as effective as S in boosting S-primed
369 mice. In macaques, both antigens were equally immunogenic and elicited neutralizing antibody
370 levels that exceeded those of convalescent patients.

371 The CoV surface glycoprotein S, and specifically its RBD domain, mediates receptor binding
372 and cell entry and is the candidate antigen for almost all vaccines in development or worldwide
373 distribution. Several studies have associated antibody-dependent enhancement (ADE) of SARS-
374 CoV infection with CoV S peptides showing high antigenic variability as the virus evolves [36],
375 while convalescent patient-derived RBD-specific antibodies effectively prevented infection in
376 rhesus macaques [3, 37] [38, 39]. These studies underscore the importance of judicious epitope
377 selection in vaccine design. Similarly, Chen et al. [40]reported on the superior safety and
378 immunogenicity in mice of an adjuvanted recombinant SARS-CoV RBD peptide over the
379 adjuvanted full-length SARS S protein, as manifested by higher neutralization antibody levels

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380 and reduced eosinophilic pulmonary infiltrates without mortality following lethal viral challenge.
381 Other studies suggest the value of including non-S epitopes, including the highly conserved
382 immunogenic domains of N [41], in the vaccine to better mimic the responses elicited following
383 natural infection and reduce the risk of ADE. For instance, mice primed with an adjuvanted
384 SARS-CoV N-based vaccine delivered intranasally and boosted intramuscularly with N-
385 expressing vaccinia Ankara virus exhibited both systemic and mucosal immune responses and
386 higher T-cell proliferative and IL-2 responses as compared to animals subjected to a homologous
387 parenteral prime-boost regimen [42]. Raghuwanshi et al. reported high anti-N IgA and IgG
388 responses in mice elicited by an intranasal DNA vaccine targeting SARS-CoV N protein
389 delivered in biotinylated chitosan nanoparticles designed for selective uptake by resident
390 dendritic cells [43]. Clinical studies have identified immunodominance of non-S circulating
391 $CD8^+$ T-cell epitopes in sera of patients who had recovered from mild COVID-19 [31].
392 Similarly, Le Bert et al. identified N and non-structural protein (NSP)-targeted T-cell responses
393 among patients recovering from COVID-19, as well as memory SARS nucleoprotein (NP)-
394 specific T-cell immunity, cross-reactive with the SARS-CoV-2 NP, among patients infected in
395 the 2003 outbreak [30]. Others identified codominance of SARS-CoV-2 M, S and N protein
396 $CD4^+$ T-cell reactivity and $CD8^+$ memory T-cell responses in convalescent patients who had
397 suffered from mild to moderate COVID-19 [29]. T cells targeting other viral proteins were also
398 identified. Comparative studies will be required to conclusively determine if the inclusion of
399 multiple antigens in a single vaccine formulation broadens virus-neutralizing activity as
400 compared to single-epitope vaccines, if it provides more extensive protection against reinfection,
401 and if it impacts disease pathology.

402 Despite the centrality of neutralizing antibodies, reported correlations between IgG titres and
403 COVID-19 severity are conflicting [44, 45], suggesting a pivotal role of cellular immune
404 responses in vaccine-induced protection. Numerous studies analysing T-cell responses among
405 COVID-19 patients, including some with undetectable antibody responses, identified enhanced

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406 Th1 cytokine levels, i.e., IFN-γ, IL-2 and TNF-α [29, 31, 46], generally within two weeks of
407 symptom onset. Nevertheless, the contribution of and balance between humoral and cellular
408 immunity still requires comprehensive clinical investigations.

409 In addition to the potential clinical benefits of careful selection and combination of viral
410 epitopes, the subunit vaccine carries several technical advantages over inactivated, attenuated or
411 viral vector vaccines, including the possibility of mass-production in dedicated fermenters and
412 no risk of contamination with residual pathogenic material. Another advantage for the subunit
413 vaccine platform is the possibility to quickly adapt the vaccine to upcoming variants by changing
414 the RBD sequence only.

415 In the context of the ongoing COVID-19 pandemic, with the need for large supplies of easy-to-
416 use vaccines, oral inoculation is a user-friendly mass-vaccination strategy. Given that SARS-
417 CoV-2 is transmitted primarily via respiratory droplets [47], robust mucosal immunity might
418 improve protection against nasal and/or oral virus entry [48] and may accelerate the development
419 of herd immunity [49]. Moreover, mucosal immunity blocks viral entry, subsequently lowering
420 the risk of infection. In addition, this route promises to overcome significant technical constraints
421 related to vaccine administration, including the avoidance of needles as an extra device to be
422 distributed, being more comfortable for needle-phobic people to use, and the ability to self-
423 medicate, especially in developing countries.

424 The integration of the highly conserved N protein may contribute to group-common immunity
425 against SARS-CoV-2 variant viruses [50]. In addition to issues of convenience of use, an oral
426 boost option may be advantageous to those patients who suffered adverse reactions to previous
427 doses of an injected vaccine.

428 The limitations of this study included relatively diverse immune responses between animals,
429 which may be attributed to the technical and physiological differences between animals at the
430 time of administration, e.gsaliva conditions, technical oral delivery to the rodents, and others.

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431 Overcoming such obstacles may be achieved by improved formulation of the vaccine, enabling
432 longer exposure to the vaccine.

433 Taken together, the oral multi-epitope SARS-CoV-2 vaccine triggered versatile adaptive immune
434 responses, which are expected to provide protection against viral infection and which should be
435 useful for boosting immunity in those immunized with injected vaccines.

436

437 **Acknowledgements:** Authors would like to thank Rivka Zaibel and Dr. Liat Hershkovitz
438 (ADRES, Advanced Regulatory Services) for their kind help and discussion.

439 Authors would like to thank Yehudit Posner for aiding, editing and proofing this manuscript.

440 Authors would like to thank Dr. Ron Ellis and Prof. Itamar Shalit for their consultation and
441 contribution to this work.

442 **Declaration of interest statement:**

443 The authors are in collaboration with MigVax Ltd in the development of its vaccine.

444 **Funding source:**

445 The Israel Innovation Authority and MigVax Ltd.

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590 **Figure Captions**

591 **Figure 1. Heterologous prime-boost vaccination schedule.**

592 Ten 8-week-old Sprague-Dawley rats per treatment group were injected subcutaneously with
593 full-length S1 subunit administered once (mixed 1:1 (v/v) with complete Freund's adjuvant
594 (FCA)) or twice (second dose mixed with incomplete Freund's adjuvant (IFA)) at a 14-day
595 interval. Two weeks after the last priming dose, oral MigVax-101 was administered once or
596 twice at a 14-day interval. Control rats received one or two subcutaneous doses of S1, followed
597 by one or two oral doses of phosphate buffer or a third dose of S1(IFA). Blood samples were
598 collected 14 days after administration of the last booster dose, after which animals were
599 euthanized, and BALF and spleens were collected.

600 **Figure 2. Oral immunization induces anti-S1 IgG and IgA antibodies**

601 Mice (BALBc, 8-week-old, 5 males and 5 females per treatment group) were inoculated orally or
602 by gavage on days 0, 14, and 28. Oral vaccine is a combination of S1, LTB-NN, LTB-NC and
603 free LTB, at either a high dose (oHD-LTB) or low dose (oLD-LTB) or high dose without free
604 LTB (oHD). Mice received the high dose with free LTB (gHD-LTB) by gavage. Control mice
605 were treated with an oral dose of PBS. Blood samples were drawn on 26 and 49 days after the
606 first immunization for determination of IgG levels. After sacrifice, wet faeces samples were
607 collected from the colon for determination of IgA levels. (A) Anti-S1 IgG titres measured 21
608 days after the third vaccination (day 49). (B) Anti-S1 IgG levels measured 26 and 49 days after
609 the first immunization. (C) Anti-S1 IgA levels in faeces samples, measured 49 days after the first
610 immunization. Statistical tests performed to determine p-values are indicated in the figure.

611 **Figure 3. Neutralization potency following oral immunization**

612 Mice (BALBc, 8-week-old, 5 males and 5 females per treatment group) were inoculated orally or
613 by gavage on days 0, 14, and 28, with either high dose (oHD-LTB) or low dose (oLD-LTB) or

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614 high-dose vaccine without free LTB (oHD) per Figure 2. Mice received the high dose with free
615 LTB (gHD-LTB) by gavage. Control mice were treated with an oral dose of PBS. Sera were
616 diluted 10 fold and assessed for neutralizing activity using the cPass neutralization assay. The y-
617 axis corresponds to the observed percentage of the binding inhibition of ACE2-RBD. The
618 neutralization assay was performed in triplicate for each mouse serum; values show mean ±
619 standard deviation. Student's t-test was performed to determine p-values.

620 **Figure 4. Oral immunization induces cellular responses**

621 Mice (BALBc, 8-week-old, 5 males and 5 females per treatment group) were inoculated orally or
622 by gavage on days 0, 14, and 28, with either high dose (oHD-LTB) or low dose (oLD-LTB) or
623 high dose vaccine without free LTB (oHD) per Figure 2. Mice received the high dose with free
624 LTB (gHD-LTB) by gavage. Control mice were treated with an oral dose of PBS. Mice were
625 sacrificed 49 days after the first immunization and spleens were harvested. Harvested
626 splenocytes (3.75×10^6 cells) were incubated overnight with 10 μg/mL N, 10 μg/mL S1 or with
627 PMA (5 ng/mL)-ionomycin (1 μg/mL) (positive control) or cell medium (negative control). (A)
628 Cell proliferation following N induction was determined using ELISPOT plates and ALAMAR
629 blue. Fluorescence was measured (560 nm/590 nm), and blank-well readings were subtracted
630 from readings of all experimental wells. (B) IFN-γ-secreting T cell counts following N induction
631 were determined by staining samples with anti-IFN-γ-peroxidase antibodies and substrate per
632 manufacturer's instructions. Spots were counted with an ELISpot reader. (C-D) Levels of
633 secreted IL-2, IFN-γ, IL-4 and IL-10 cytokines, following (C) N induction or (D) S induction,
634 were determined by ELISA. Statistical tests performed to determine p-values are indicated in the
635 figure.

636

637 **Figure 5. Humoral and mucosal responses of antibodies generated following heterologous**
638 **prime-booster SARS-CoV-2 vaccination**

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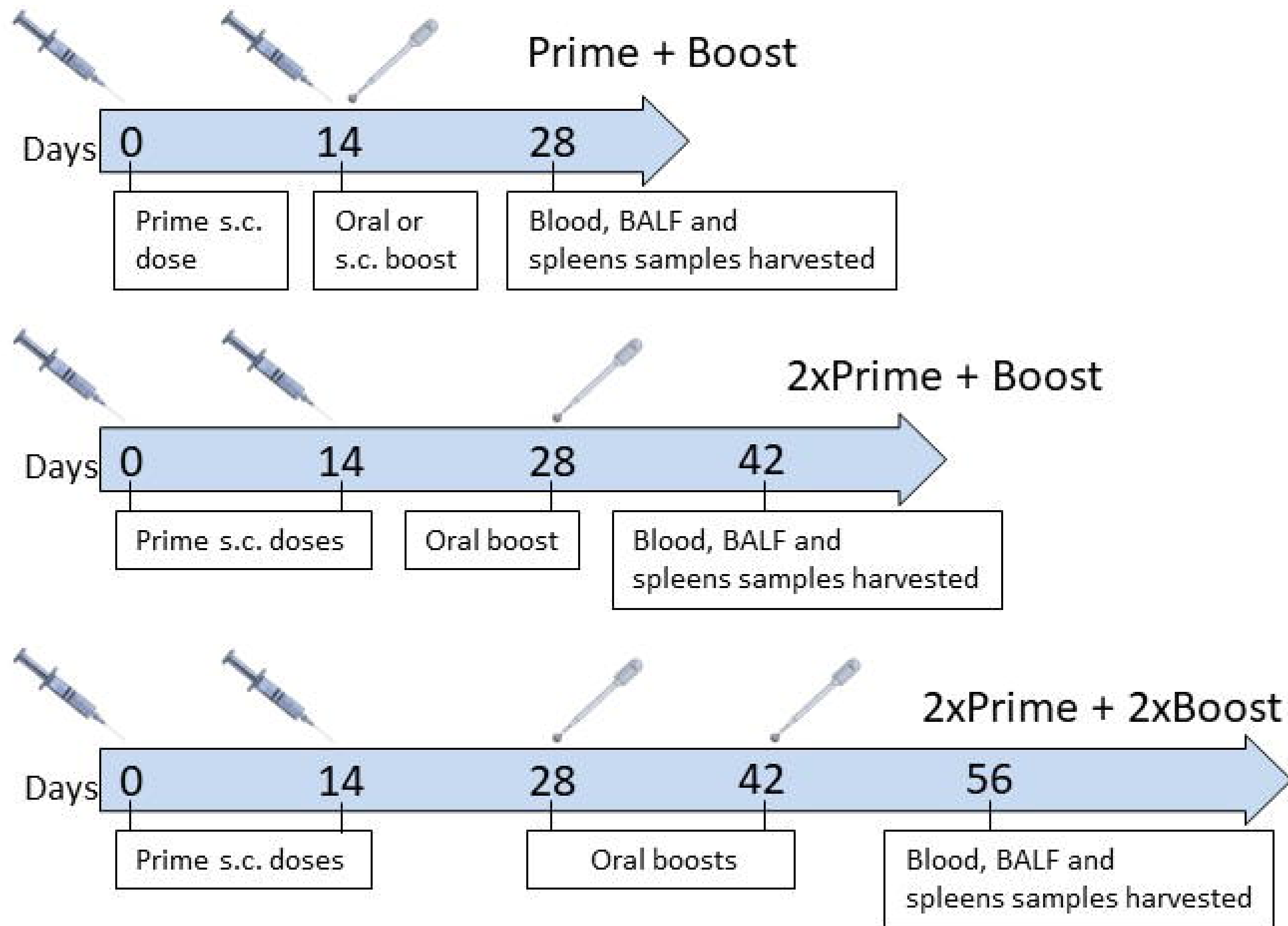
639 Sprague Dawley rats (8-weeks-old, 10 per treatment group) were injected subcutaneously once
640 or twice at a 14-day interval with adjuvanted S1 subunit. Two weeks after the last priming dose,
641 oral MigVax-101 was administered once or twice at a 14-day interval. Control rats received one
642 or two injections of S1, followed by one or two oral doses of PBS or a third injected dose of S1.
643 (A) Anti-S1 IgG levels determined by ELISA in sera samples collected 14 days after the last
644 booster dose. (B) Anti-S1 IgA levels in broncheo-alveolar lavage fluid, determined by ELISA 14
645 days after the last booster dose. Statistical tests performed to determine p values are indicated in
646 the figure.

647 **Figure 6. Pseudo-virus neutralization in Vero E6 cells following heterologous prime-**
648 **booster SARS-CoV-2 vaccination**

649 Sprague Dawley rats (8-weeks-old, 10 per treatment group) were injected subcutaneously once
650 or twice, at a 14-day interval, with adjuvanted S1 subunit. Two weeks after the last priming dose,
651 oral MigVax-101 was administered once or twice, at 14-day interval. Control rats received one
652 or two injections of S1, followed by one or two oral doses of PBS or a third injected dose of S1.
653 Presented are results from two-fold serial dilutions of heat inactivated serum collected 14 days
654 after the third immunization were incubated with pseudo-SARS-CoV-2 virus. The y-axis
655 corresponds to the titter in rat sera of antibodies neutralizing virus infection of Vero E6 cells.
656 The neutralization assay was performed in triplicates for each rat and the presented values show
657 the mean ± standard deviation. Statistical tests performed to determine p values are indicated in
658 the figure.



Prime + Boost
Days
0
14
28
Prime s.c. dose
Oral or s.c. boost
Blood, BALF and spleens samples harvested
2xPrime + Boost
Days
0
14
28
42
Prime s.c. doses
Oral boost
Blood, BALF and spleens samples harvested
2xPrime + 2xBoost
Days
0
14
28
42
56
Prime s.c. doses
Oral boosts
Blood, BALF and spleens samples harvested

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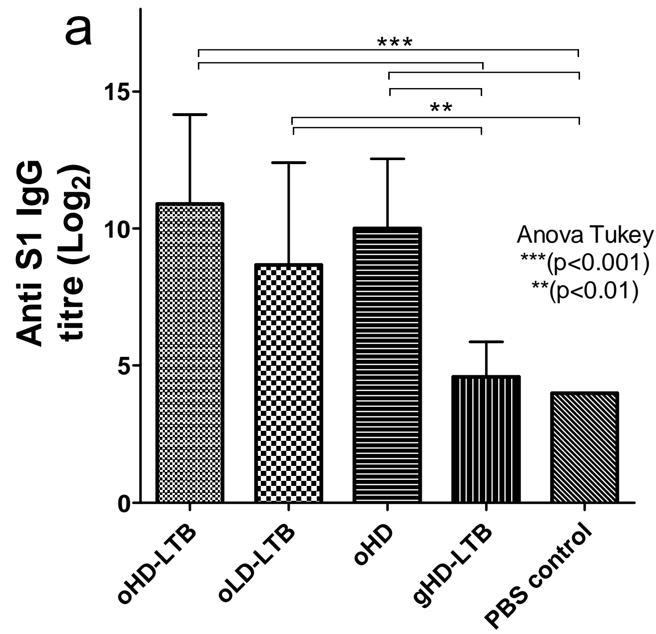

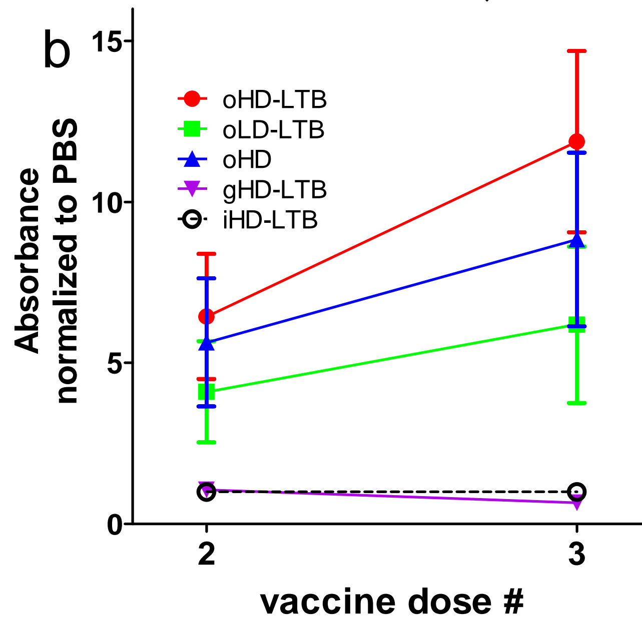

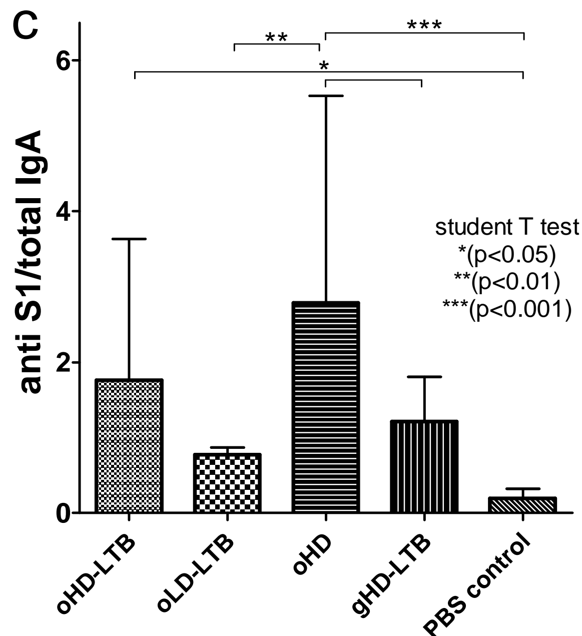

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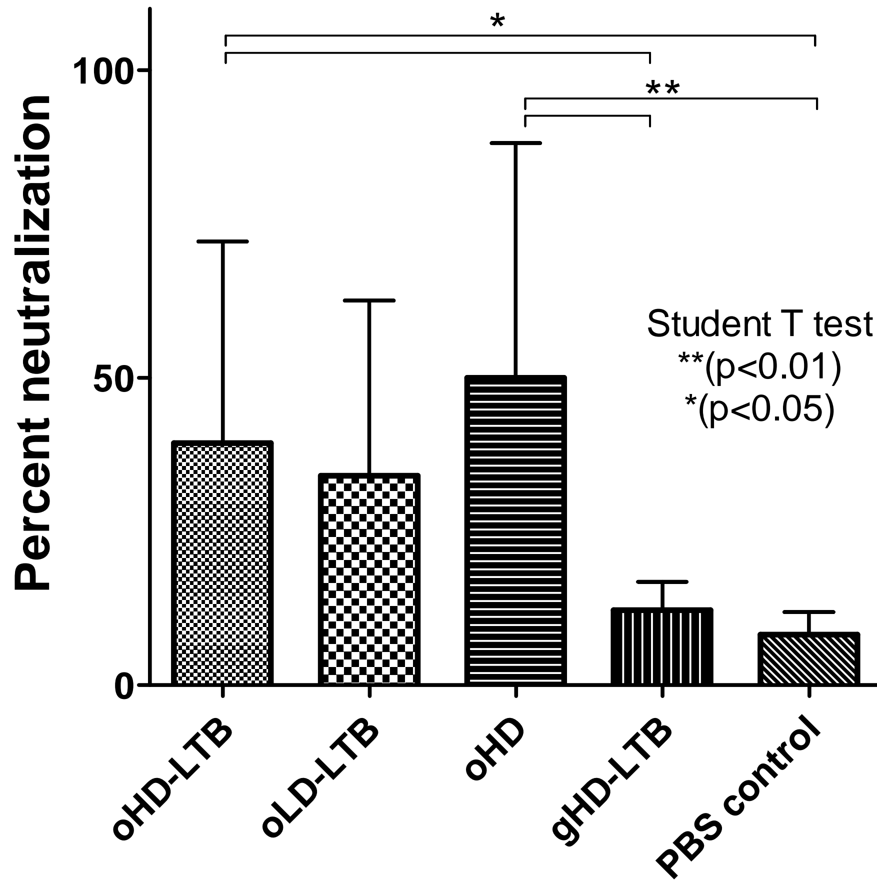
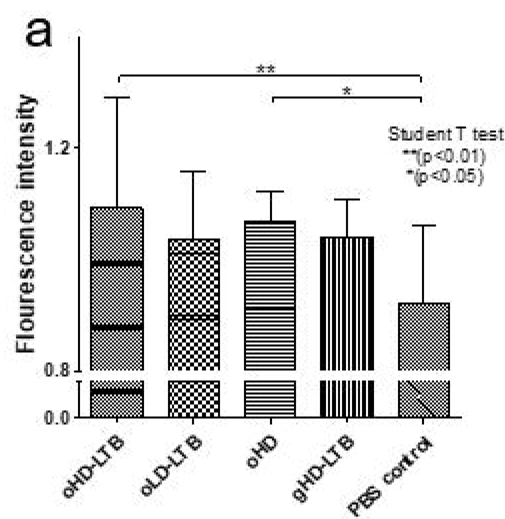
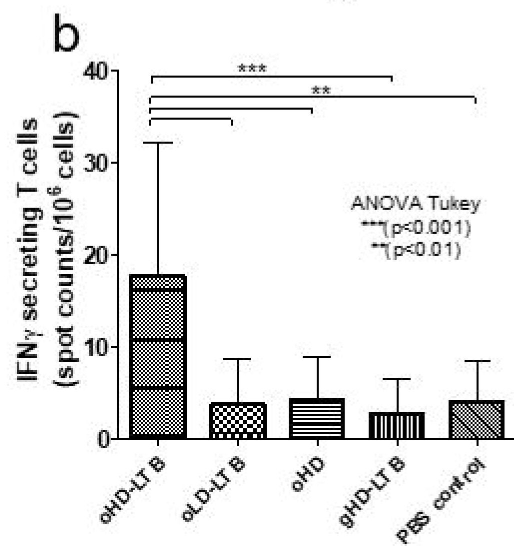
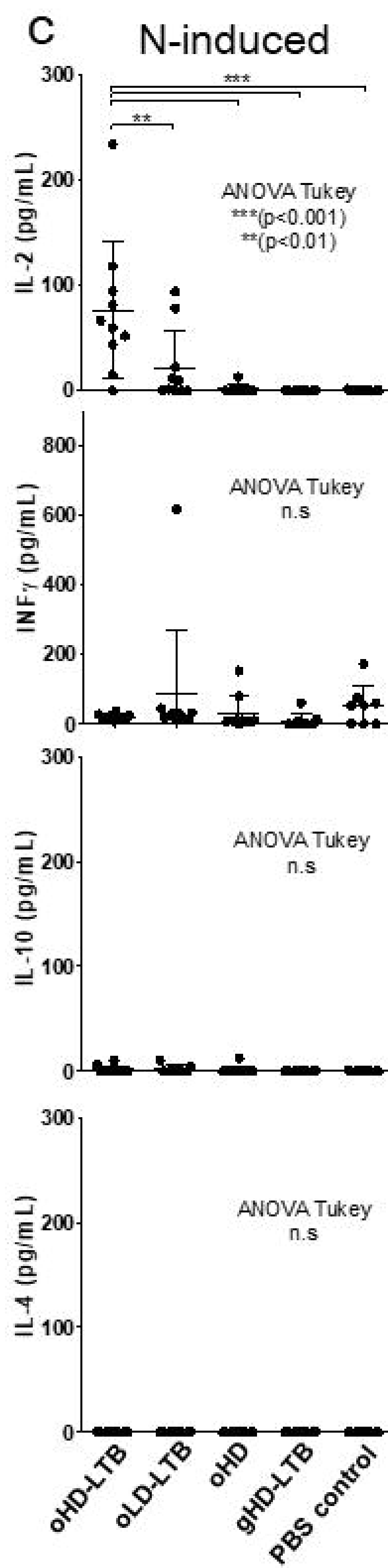
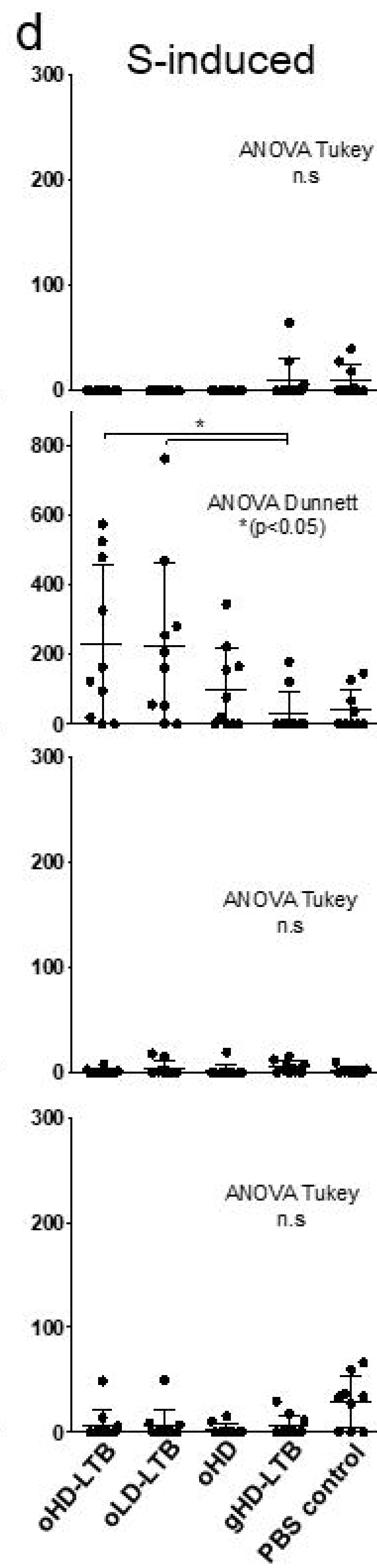



a
Flourescence intensity
1.2
0.8
0.0
Student T test
**(p<0.01)
*(p<0.05)
oHD-LTB
oLD-LTB
oHD
gHD-LTB
PBS control


b
IFNγ secreting T cells
(spot counts/10^6 cells)
ANOVA Tukey
***(p<0.001)
**(p<0.01)
oHD-LTB
oLD-LTB
oHD
gHD-LTB
PBS control


c
N-induced
IL-2 (pg/mL)
ANOVA Tukey
***(p<0.001)
**(p<0.01)
INFγ (pg/mL)
ANOVA Tukey
n.s
IL-10 (pg/mL)
ANOVA Tukey
n.s
IL-4 (pg/mL)
ANOVA Tukey
n.s
oHD-LTB
oLD-LTB
oHD
gHD-LTB
PBS control


d
S-induced
ANOVA Tukey
n.s
ANOVA Dunnett
*(p<0.05)
ANOVA Tukey
n.s
ANOVA Tukey
n.s
oHD-LTB
oLD-LTB
oHD
gHD-LTB
PBS control

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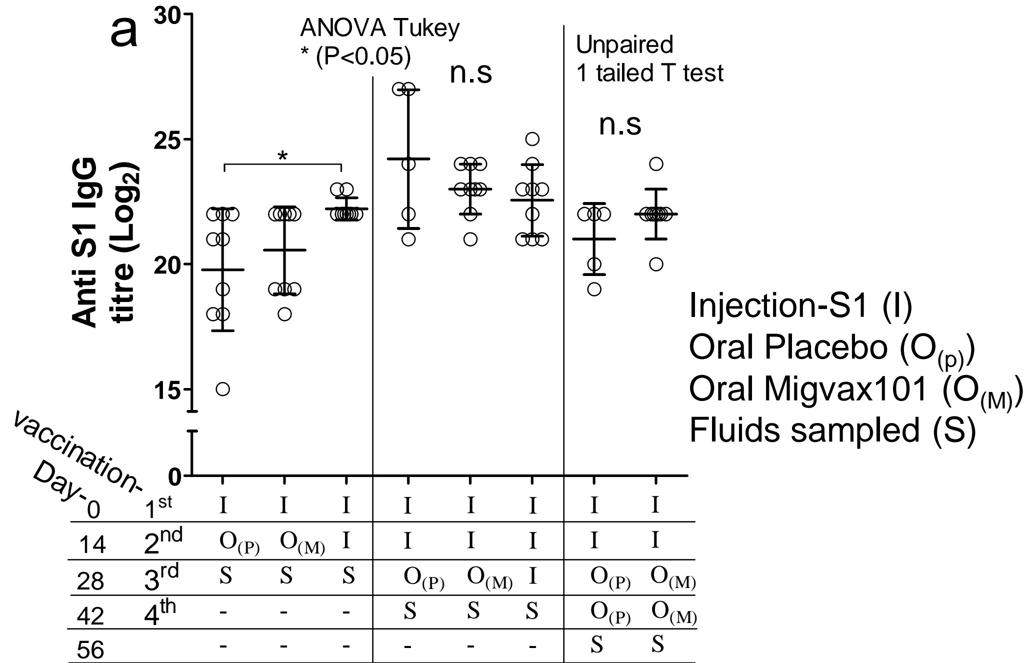

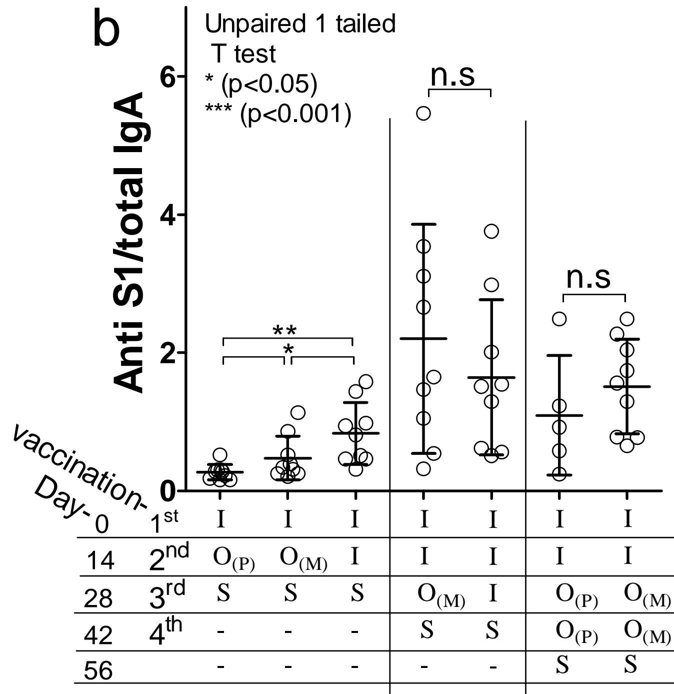

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