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Receptor-binding domain-based SARS-CoV-2 vaccine adjuvanted with cyclic di-adenosine monophosphate enhances humoral and cellular immunity in mice

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ABSTRACT

Novel adjuvants are highly desired to improve immune responses of SARS-CoV-2 vaccines. This work reports the potential of the stimulator of interferon genes (STING) agonist adjuvant, the cyclic di-adenosine monophosphate (c-di-AMP), in a SARS-CoV-2 vaccine based on the receptor binding domain (RBD). Here, mice immunized with two doses of monomeric RBD adjuvanted with c-di-AMP intramuscularly were found to exhibit stronger immune responses compared to mice vaccinated with RBD adjuvanted with aluminium hydroxide (Al(OH)3) or without adjuvant. After two immunizations, consistent enhancements in the magnitude of RBD-specific IgG antibody response were observed by RBD+c-di-AMP (mean:15360) compared to RBD+Al(OH)3 (mean:3280) and RBD alone (n.d.). Analysis of IgG subtypes indicated a predominantly Th1-biased immune response (IgG2c, mean:14480; IgG2b, mean:1040, IgG1, mean:470) in mice vaccinated with RBD+c-di-AMP compared to a Th2-biased response in those vaccinated with RBD+Al(OH)3 (IgG2c, mean:60; IgG2b: n.d.; IgG1, mean:16660). In addition, the RBD+c-di-AMP group showed better neutralizing antibody responses as determined by pseudovirus neutralization assay and by plaque reduction neutralization assay with SARS-CoV-2 WT. Moreover, the RBD+c-di-AMP vaccine promoted IFN-γ secretion of spleen cells cultures after RBD

stimulation. Furthermore, evaluation of IgG-antibody titers in aged-mice showed that di-AMP was able to improve RBD-immunogenicity at old-age after 3 doses (mean:4000). These data suggest that c-di-AMP improves immune responses of a SARS-CoV-2 vaccine based on RBD, and would be considered a promising option for future COVID-19 vaccines.

KEYWORDS

SARS-COV-2, VACCINES, ADJUVANT, C-DI-AMP, IMMUNE RESPONSE

INTRODUCTION

COVID-19 is a respiratory disease caused by a novel SARS-CoV-2 virus found in Wuhan, China, in December 2019[1]. Since then, COVID-19 has rapidly spread, significantly impacting global public health. To date, a number of vaccines were quickly developed and approved for emergency use at unprecedented times[2]. However, there is still an urgent need for further vaccine development, particularly in low- and middle-income countries, as well as new approaches to improve vaccine efficacy to cover emerging variants around the world.

In view of the wide range of vaccine development strategies currently under consideration, recombinant proteins represent a feasible solution for rapid SARS-CoV-2 vaccine manufacture due to its relative low cost and ease in production. Accordingly, the RBD of the spike protein which binds to the human receptor angiotensin-converting enzyme 2 (ACE2), it has been proposed as an interesting recombinant antigen for vaccines[3]. RBD-based vaccines are attractive, since infected

patients have been shown to exhibit potent neutralizing antibodies against RBD epitopes that correlate strongly with effective protection against SARS-CoV-2 infection[4–6].Although new SARS-CoV-2 variants also have mutations in the RBD domain, it has been demonstrated that antibodies from animals vaccinated with RBD from SARS show cross-reactivity and neutralization against SARS and SARS-CoV-2[7] and other sarbecoviruses[8]. Moreover, novel recombinant RBD-based vaccines are in preclinical and clinical trials and have shown to induce protective responses against SARS-CoV-2[9].

Current new strategies to obtain more effective COVID-19 vaccines have been included the incorporation of potent adjuvants able to induce immunomodulation to activate T-cell responses. The Al(OH)3 is widely used adjuvant in vaccines to induce antibodies and Th2 responses[10]. However, it is not effective in inducing strong activation of T helper 1 (Th1) and cytotoxic responses that are crucial to fight viral infection[11].

In recent times, many novel adjuvants able to activate T-cell responses are being investigated, particularly those that can be detected by pattern recognition receptors (PRRs). Within these, the cyclic dinucleotides are compounds recognized by the STING receptor, a transmembrane intracellular cytoplasmic protein, which promote the activation of immune system to induce potent humoral immune and T-cell responses[12,13].

Recent studies have shown encouraging protective results in mice vaccinated with the c-di-AMP adjuvanted formulations against influenza and other infectious

diseases principally by mucosal route[14–18]. In view of the excellent adjuvant capacity of the c-di-AMP, here it was investigated the promising role of c-di-AMP and its potential use in intramuscular COVID-19 vaccines.

Therefore, humoral and cellular immune responses in mice vaccinated with RBD adjuvanted with c-di-AMP were evaluated and compared with those induced by RBD or RBD adjuvanted with Al(OH)3. Consequently, RBD-specific IgG antibodies elicited in vaccinated young and aged-mice were measured. Next, the ability of antibodies to neutralize both, SARS-CoV-2 live Wild Type B.1 and a VSV pseudovirus carrying SARS-CoV-2 Spike-D614G. Furthermore, the capacity to promote cellular immune responses in young mice was studied.

The findings presented here have important implications for understanding the role of the c-di-AMP adjuvant in SARS-CoV-2 vaccines.

METHODS

Mice vaccination

Young female C57BL/6 mice (n=5) (3-month-old) were immunized by intramuscular route with 20 μg of RBD alone or formulated with cyclic di-adenosine monophosphate (10 μg) or aluminium hydroxide gel (250 μg) (Invivogen), on days 1 and 21. A control PBS group was included. Aged-female C57BL/6 mice (n=4, 20-month-old) were immunized by intramuscular route with 20 μg of RBD formulated with cyclic di-adenosine monophosphate (10 μg) on days 1, 21 and 42. Serum samples were taken before the booster vaccinations and at the end of the protocol.

The recombinant monomeric RBD produced in *Pichia Pastoris* was kindly provided by Argentinian Anti-Covid Consortium, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, and used in previous works[19,20]. All animals were cared in accordance with the Guiding Principles in the Care and Use of Animals of the US National Institute of Health. All procedures were approved by the Institutional Animal Care and Use Committee of the School of Medical Science, Universidad Nacional de Cuyo (Protocol approvals, Nº 186/2020, Nº 203/2021).

Determination of antibody responses by ELISAs

SARS-CoV-2 RBD-specific IgG and IgG1, IgG2b and IgG2c antibodies titers and IgG total were measured by enzyme linked immunosorbent assay (ELISA). Briefly, 96-well plates were coated with 100 µl/well of recombinant SARS-CoV-2 RBD protein (2 µg/ml) and incubated overnight at 4 ºC. All the washes were performed with PBS containing 0.05% Tween-20. After incubation and washes, plates were blocked by 2% BSA in PBS for 2 h at 37 ºC. After washing, two-fold serial dilutions of mouse sera were added into the wells for 2 h at 37 ºC. Afterwards, wells were washed and secondary mouse-specific IgG-detection antibodies were added, such as HRP-conjugated goat anti-mouse IgG (Thermofisher), biotinylated rat anti-mouse IgG1, or biotinylated rat anti-mouse IgGc, or biotinylated rat anti-mouse IgG2b (BD Pharmingen, New Jersey, USA) and then incubated for 1 h at 37 ºC. For plates where the biotinylated antibodies were used, 100 µl/well of peroxidase conjugated streptavidin (BD Pharmingen, New Jersey, USA) was added to each well, and incubated at room temperature for 1 h. After washing, colorimetric reactions were

performed by the addition of a 3´,3´,5´,5´-tetramethylbenzidine (TMB) solution (Promega, USA) and the reaction was stopped by H2SO4. The optical density was measured at 450 nm, by an ELISA microplate reader (Multiskan EX; Thermo Scientific, Pittsburgh, USA). The endpoint titer, were expressed as the reciprocal of the highest dilution of serum that yielded an optical density 2 times above the mean value of the pre-immune sera. The lower limit of detection was at a dilution of 1:100. Mice who did not elicit specific antibodies titers were arbitrary graphed as 1:50.

Total mouse IgG was measured by ELISA sandwich. Briefly, 96-well plates were coated with goat anti-Mouse IgG antibody (Thermofisher) and incubated overnight at 4 ºC. After incubation and washes with PBS containing 0.05% Tween-20, plates were blocked by 2% BSA in PBS for 2 h at 37 ºC. After washing, a 1/10000 dilution of individual sera were added into the wells for 2 h at 37 ºC. Afterwards, wells were washed and a secondary goat anti-Mouse IgG antibody HRP-conjugated (Thermofisher) was added, and incubated for 1 h at 37 ºC. After washing, colorimetric reactions were performed by the addition of a 3´,3´,5´,5´-tetramethylbenzidine (TMB) solution (Promega, USA) and the reaction was stopped by H2SO4. The optical density was measured at 450 nm, by an ELISA microplate reader (Multiskan EX; Thermo Scientific, Pittsburgh, USA). Concentration was calculated using an IgG standard curve.

Determination of cytokines by ELISAs

The cytokines in cell culture supernatants of splenocytes stimulated with RBD (10 μg/ml final concentration) for 72 h, without stimulation, or stimulated with Concanavalin A (10 μg/ml) were determined by ELISA assay using OptEIA Set Mouse IFN-γ, IL-4 and IL-10 (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. The values of un-stimulated samples were considered background and subtracted from the RBD- specific responses measured from for each individual mouse.

Intracellular cytokine staining

Murine splenocytes were stimulated for a total of 24 h with RBD (10 μg/ml final concentration) or media only (unstimulated control), and Brefeldin A/monensin (BD Biosciences, USA) were added according to the manufacturer's instructions for the final 5 h. Then, the cells were stained with anti-CD8+conjugated with FITC, and anti-CD4+conjugated with APC (BD Biosciences, USA) for T cell surface receptors. The following day, the cells were stained with anti-IFN-γ conjugated with PE and anti-IL4 conjugated with PE.Cy7 (BD Biosciences, USA). Sample acquisition was performed on a FACSAria III (BD Biosciences, USA)). The values of un-stimulated samples were considered background and subtracted from the RBD-specific responses measured from for each individual mouse (n=3).

Neutralizing Antibodies by pseudovirus neutralization assay

SARS-CoV-2 neutralization was assessed with spike-pseudotyped virus[21]. For this assay, serum from mice obtained at 42 days after prime immunization were used.

Briefly, Vero ACE2 CCL81 cells were seeded in a 24 well culture plate in a final volume of 250 µl of alpha-MEM containing 10% FBS and infected with 10^2 PFU of pseudovirus VSVg-SpikeD614G-GFP (generously provided by Dr. Sean Whelan, St. Louis, MO, USA) pre-incubated with 1:2 and 1:20 dilutions of serum for 1h at 37°C. Final dilutions are calculated take into consideration the final dilution with pseudovirus. After 24h, the cells were fixed with 2% paraformaldehyde and evaluated by fluorescence microscopy to visualize the infected fluorescent cells (qualitative assay)[21]. For quantified assay, the infected GFP- fluorescent cells were washed, fixed in 1% paraformaldehyde, and analysed with a Becton Dickinson FACSAria II. The populations were quantified based on fluorescent labelling and the FSC/SSC profile. The percentage calculation was obtained by referring to a positive infection control, without sera, as 100% infection.

Neutralizing Antibodies by Plaque reduction neutralization test with live SARS-CoV-2

Serum samples from mice were tested for their neutralization ability against SARS-CoV-2 live Wild Type B.1 (GISAID, accession number ID: EPI_ISL_499083) by the plaque-reduction neutralization test (PRNT), as was previously described [22]. Briefly, the virus neutralization test was performed with Vero Cl76 cells (ATCC CRL-1587) that were seeded in 24-well plates 48 h before infection. Plasma samples were heat-inactivated by incubation at 56°C for 20 minutes and centrifuged at 10,000 rpm 30 minutes before use. Treated samples were two-fold diluted and then an equal volume of virus stock containing 100 plaque forming units (PFU) was added to each corresponding well until reaching final dilutions ranging from 1/10 to 1/320. Sample

dilutions-virus were incubated at 37°C for 60 min in a 5% CO2 incubator and then added to each well of the cell culture plate. After incubation for 1 hour at 37°C in a 5% CO2 incubator, cells were incubated with 0.5% agarose with DMEM supplemented with 2% FBS for 4 days at 37°C in a 5% CO2 incubator. After 4 days, cells were fixed and inactivated using a 10% formaldehyde/PBS solution and stained with 1% crystal violet. Neutralizing antibody titers corresponded to the maximum dilution of plasma that neutralized 50% of the PFU, compared with PFU from the viral controls included in the test.

ELISPOT

For IFN-γ ELISPOTs, splenocytes from mice of a same group were pooled and cultured (4 x 10^5 cells/well, 6 replicates) in ELISPOT plates, and incubated in the absence or presence of RBD (10 μg/ml) or Concanavalin A (5 μg/ml) for 18 h, at 37°C and 5% CO_2. After 18 h IFN-γ ELISPOT kit (BD Pharmingen, USA) were used according to the manufacturer's instructions. The plates were analyzed and counted using a digital microscope M-DIG7 (Gadnic). The results were expressed to 1 x 10^6 cells.

Statistical analysis

Results are expressed as the mean±SEM. Statistical differences were calculated by Student t-test, non-parametric Mann–Whitney test, one-way ANOVA followed by Sidak's Multiple comparison, and two-way analyses of variance (ANOVAs) followed by Tukey's Multiple comparison Test. P values <0.05 were considered significant: * *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.*

RESULTS

RBD adjuvanted with c-di-AMP *promotes enhanced antibody response in young mice*

Young mice were immunized intramuscularly in a two-dose regimen (days 0 and 21) with vaccines containing RBD alone, RBD+Al(OH)3, or RBD+c-di-AMP. Humoral immune responses elicited by the formulations, RBD-specific IgG and IgG subclasses (IgG1, IgG2c and IgG2b), were evaluated by ELISA assays.

It was found that mice vaccinated with RBD without adjuvants did not induce detectable anti-RBD IgG titers. Mice vaccinated with RBD+Al(OH)3 showed detectable anti-RBD IgG titers after two immunizations (mean: 3280), but it was undetectable with a single dose on day 21. In contrast, mice immunized with the RBD+c-di-AMP showed anti-RBD IgG titers at the first immunization (mean:260), and increased significantly after two immunizations (mean:15360) (Figure 1 A).

Furthermore, the analysis of IgG subclasses showed that mice vaccinated with RBD+Al(OH)3 had a skewed predominance of IgG1 titers indicating a Th2-biased immune response. In contrast, mice immunized with RBD+c-di-AMP showed a predominance of IgG2c and IgG2b over IgG1 titers, indicating mainly a Th1-biased immune response (Figure 1-C).

In order to evaluate whether the use of adjuvants may cause a systemic response and increase overall antibodies in serum, total non-specific IgG antibodies were evaluated by ELISA sandwich. It was observed that mice vaccinated with the adjuvanted formulations did not increase the total IgG levels in serum (Figure 1-B).

Antibodies from mice vaccinated with RBD+c-di-AMP show a significant neutralizing capacity

The neutralizing capacity of the antibodies was then evaluated by the pseudovirus neutralization assay and by PRNT50 with SARS-CoV-2 Wild Type B.1.

The pseudovirus neutralization assay showed that 3 of 5 sera from mice vaccinated with RBD+c-di-AMP were able to neutralize VSV-pseudotyped virus inducing less than 50% of infection at a 1:8 serum dilution, and one serum reduced the infection at a 1:80 a serum dilution. In comparison, only one serum from mice vaccinated with RBD+Al(OH)3 reduced the infection at a 1:8 serum dilution, and none at 1:80 serum dilution (Figure 2-A).

The PRNT50 showed that 2 sera from mice vaccinated with RBD+c-di-AMP had detectable nAbs against SARS-CoV-2 WT at serum dilutions of 1:10 and 1:80, and a mouse vaccinated with RBD+Al(OH)3 showed neutralizing antibodies at a serum dilution of 1:20 (Figure 2-B). All the sera from RBD alone and PBS groups did not neutralize.

Cellular immune response due to RBD+c-di-AMP vaccination

Although the RBD protein is of interest in inducing protective antibody responses, it is less clear how it could contribute to activate cellular immunity. To measure T cell-mediated immune responses, Th1 cytokines as IFN-γ, and Th2 cytokines as IL-4, and IL-10 were determined by ELISA assays.

It was observed that mice vaccinated with RBD+c-di-AMP produced higher levels of IFN-γ and IL-4 compared to mice vaccinated with RBD or RBD+Al(OH)3. Nevertheless, only the level of IFN-γ was statistically significative suggesting a predominantly Th1-biased response (Fig. 3-A). The secretion of IL-10 was poor and there was no difference between the groups (data not shown). To further evaluate specificity of the cytokine production, we performed flow cytometry assays to evaluate CD4+ and CD8+ T cells producers of IFN-γ- and IL-4. It was observed that the RBD-specific IFN-γ+CD8+ T cell response trended higher in mice vaccinated with RBD+c-di-AMP. In addition, the secretion of IFN-γ by CD4+ T cell trended similar in the c-di-AMP and Al (OH)3 groups (Fig. 3-B). Moreover, the analysis of IFN-γ-ELISPOT showed that the use of c-di-AMP adjuvant elicited higher IFN-γ spots compared to RBD alone (Supplementary Fig. 1).

RBD adjuvanted with c-di-AMP *is able to improve RBD-immunogenicity in aged-mice*

Taken into account that the immune response to the vaccine is affected by age and that severe COVID is more frequent in the elderly, next, it was evaluated whether c-di-AMP would be able to enhance the humoral response in aged mice. The duration of the RBD-specific IgG humoral response was investigated for 12 weeks. It was observed that aged mice after two immunizations showed detectable anti-RBD IgG titers at day 42 (mean: 300), indicating that c-di-AMP improved the RBD- specific humoral response in aged mice. To determine whether an additional dose could enhance the humoral immune response, a third dose was administered 21 days after the last immunization and it was noticed that RBD-specific IgG titers were clearly improved

by day 56(mean: 4000). Thereafter, they started to decrease, but remained detectable until sacrifice on day 84. (Figure 4).

CONCLUSIONS

The use of potent adjuvants is an interesting approach for the optimization of new vaccines. In this regard, many works highlighted the outstanding properties of the cyclic di-nucleotides which activate the intracellular STING receptor. These compounds have already shown strong adjuvant activity in animals and have been tested in clinical trials for cancer [12]. Among them, c-di-AMP has shown enhanced antigen-specific humoral and cellular immune responses in vaccines against infectious diseases, especially by mucosal route [12,15,18].

In the current study, young mice were vaccinated intramuscularly with a formulation containing the recombinant RBD segment of the spike protein[19]in conjunction with the c-di-AMP adjuvant. It was observed that supplementation of RBD with c-di-AMP resulted in substantial anti-RBD IgG titers compared to the responses elicited in mice vaccinated with RBD alone or formulated with Al(OH)3. The reduced immunogenicity obtained by the use of RBD or RBD+Al(OH)3 was in line with other studies involving similar strategies [20,23]. Analysis of IgG subclasses showed that RBD+c-di-AMP induced Th1 as well as Th2-biased antibodies, but predominantly toward a Th1-biased response[24]. These results are important in the context that the Th1 response would be preferred in SARS-CoV-2 vaccines, since a Th2-biased response has been usually associated to the risk of vaccine-associated enhanced respiratory disease (VAERD)[25].

The rationale to use STING agonists has been reported in experimental COVID-19 vaccines [26,27]. For example, Nanishi et. al used the cyclic guanosine monophosphate–adenosine monophosphate (2′3′-cGAMP), which was formulated with a recombinant RBD produced in mammalian cells. This formulation induced poor IgG levels, low IgG1, and no significant IgG2a titer in mice. The addition of Al(OH)3 to 2′3′-cGAMP enhanced humoral immune responses but maintained a Th2-biased response, and showed very low percentage of hACE2-RBD binding inhibition[27].

In this work, it was observed that the addition of c-di-AMP to RBD improved effectively nAbs responses compared with AL(OH)3. The results showed that not all mice displayed extended sera neutralization capacity, which could agree with underlying interindividual variability of immune responses, and with reported data on different neutralization levels displayed by actual COVID vaccines[28]. Now, it is clear that COVID-19 vaccines need a robust humoral as well as cell-mediated immunity.

Regarding cellular responses, several studies have described the important role of memory T cells for long-term and broad protection against coronavirus[29–33]. In view of current vaccine candidates, the supplementation of RBD with c-di-AMP would resolve the T-cell unresponsiveness of mice vaccinated with the RBD monomer. It was observed in this work that RBD+c-di-AMP induced IFN-γ secretion in splenocytes cultures, however the source of this IFN-γ remains elusive. Similar results were found in another works in which RBD-based vaccines induce mild cellular immune response [36–38].Until now, little is known in relation to T- cell responses induced by RBD. However, the epitope SARS-CoV-2 S395-404 as a major histocompatibility complex (MHC) class I-restricted epitope for the RBD-specific CD8 T cell response in

C57BL/6 mice has been recently identified [35]. Further experiments should be done to examine profoundly the T cell-responses which may provide a better scenery of the cellular immune response.

Another important factor to take into account in this work is the route of administration, which undoubtedly plays a fundamental role in the robustness of the induced immune responses. The route of immunization used here was based on the fact that the intramuscular route has been the most used in COVID-19 mass vaccination campaigns[2]. However, the intramuscular route has not been widely evaluated for the c-di-AMP adjuvant. Accordingly, c-di-AMP has been evaluated only in one work by the intramuscular route using the β-Gal antigen model, and with the combination with Al(OH)3[14]. In addition, an earlier report demonstrated by Madhun et. al. with another c-di-nucleotide, c-di-GMP used in a plant-derived H5 influenza vaccine, showed that reduced immune responses were obtained by the intramuscular route compared to the same formulation by the mucosal route [39]. With this in mind, it is plausible that better immune responses can be obtained using the mucosal rather than the intramuscular route.

Future research on formulation design, focusing on factors such as antigen load or c-di-AMP per dose, booster injections, time between doses, may contribute to improving the strength of RBD-specific immunity. However, it is important to note that this is the first work to demonstrate the capabilities of c-di-AMP given intramuscularly in mounting functional immune responses for the prevention of COVID-19. These results suggest that c-di-AMP adjuvant would be a potential candidate to design new COVID-19 and respiratory virus vaccines.

Authors contributions

MVS: conceptualization, validation, investigation, visualization, project administration supervision, resources, writing - Original Draft, Review & Editing. MJG, JPMO: investigation, validation, visualization, Writing-Review & Editing. CG, SB, BK, LS, JA, SG: investigation. HAV, DEC, MIC, JPMO, SB: Resources, Writing-Review & Editing. All authors contributed to the interpretation of the data and approved the final manuscript.

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Legends

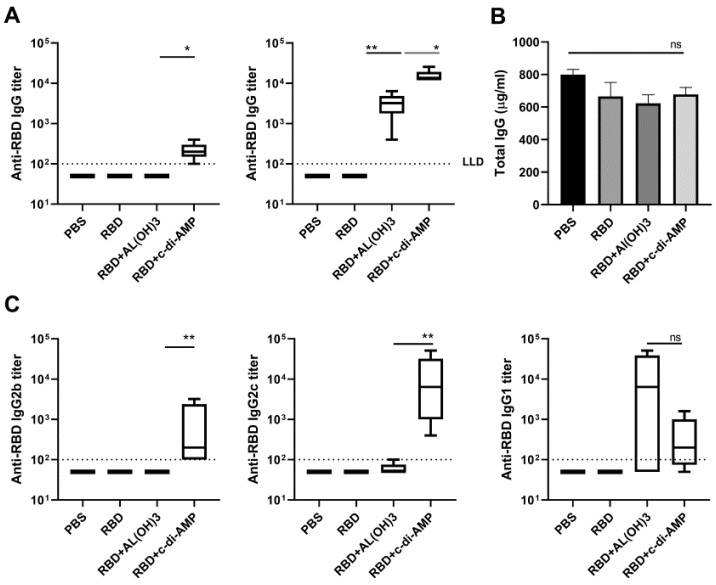

Figure 1. Humoral response of serum from vaccinated young mice. *Anti-RBD IgG antibody titers obtained on day 21 and 42 (A), total IgG (non-specific) (B) and anti-RBD IgG1, IgG2c, IgG2b antibodies titers obtained on day 42(C), were measured by ELISA using serum samples. Titers were shown as inverse dilution. Asterisks indicate comparisons to the RBD adjuvanted with Al(OH)3 group. Significance was determined*

*by Student t-test, non-parametric Mann–Whitney test. Box-and-whisker plots represent the minimum, first quartile, median, third quartile, and maximum value. LLD, lower limit of detection. *p < 0.05, **p < 0.01, ns: not significant.*

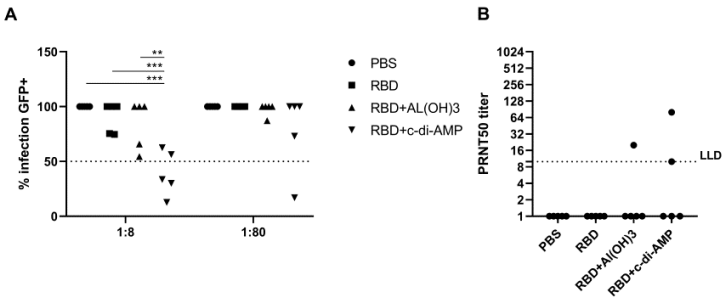

Figure 2: Neutralizing-antibody response. *Pseudoneutralization assay with pseudotyped virus carrying Spike protein using serum dilutions at 1:8 and 1:80. Percentages of GFP+infected cells were measured by flow cytometry (A). Neutralization assay against SARS-CoV-2 live Wild Type B.1, PRNT50 titers (B). Significance was determined by two-way ANOVA, Tukey's multiple comparisons test, ** p<0.01, *** p<0.001.*

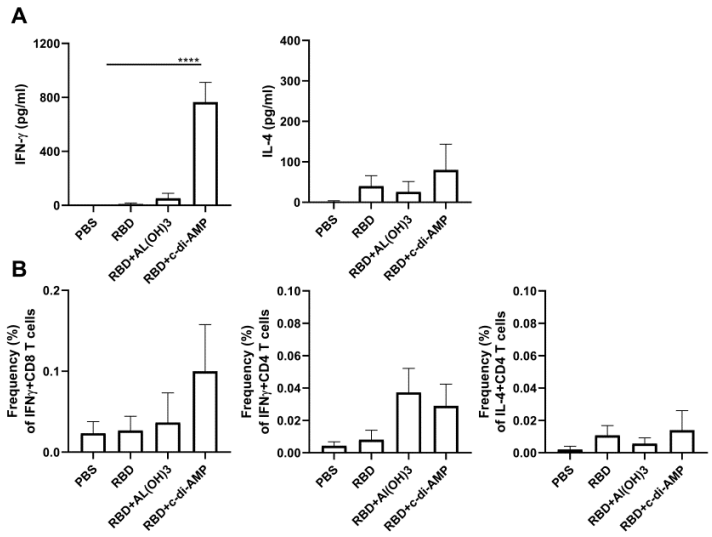

Figure 3. Characterization of the cellular immune response in immunized mice*. IFN-γ and IL-4 cytokines levels measured by enzyme-linked immunosorbent assay (ELISA) using supernatant of splenocytes stimulated for 72 h with RBD 21 days after booster (A); cytokine concentration (pg/ml). Frequency (%) of CD8+ and CD4+-cytokines producing T cells(B), measured by flow cytometry in splenocytes stimulated for 24 h. Mean value is shown. Significance was determined by one-way ANOVA, Sidak's multiple comparisons test, **** p<0.0001.*

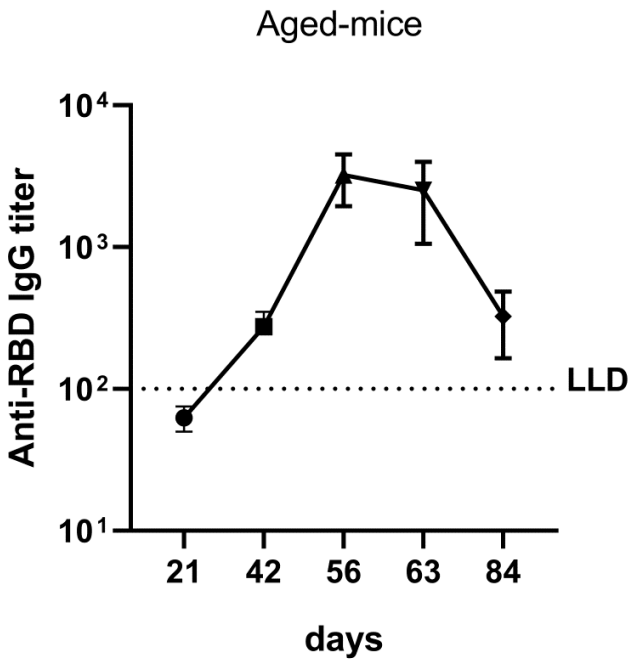

Figure 4. Durability of humoral response of aged-mice vaccinated with RBD adjuvanted with c-di-AMP*. Anti-RBD IgG titers of immunized mice during specific days of the immunization schedule, measured by ELISA.*