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COVID-19 Original Article

Comparative immunogenicity analysis of intradermal versus intramuscular administration of SARS-CoV-2 RBD epitope peptide-based immunogen *In vivo*

Naveen Yadav [1], Preeti Vishwakarma [1], Ritika Khatri, Gazala Siddqui, Amit Awasthi, Shubbir Ahmed, Sweety Samal*

Infection and Immunology Laboratory, Translational Health Science & Technology Institute, NCR Biotech Science Cluster, Faridabad, Haryana 121001, India

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ABSTRACT

COVID-19 pandemic has caused severe disruption of global health and devastated the socio-economic conditions all over the world. The disease is caused by SARS-CoV-2 virus that belongs to the family of Coronaviruses which are known to cause a wide spectrum of diseases both in humans and animals. One of the characteristic features of the SARS-CoV-2 virus is the high reproductive rate (R_0) that results in high transmissibility of the virus among humans. Vaccines are the best option to prevent and control this disease. Though, the traditional intramuscular (IM) route of vaccine administration is one of the effective methods for induction of antibody response, a needle-free self-administrative intradermal (ID) immunization will be easier for SARS-CoV-2 infection containment, as vaccine administration method will limit human contacts. Here, we have assessed the humoral and cellular responses of a RBD-based peptide immunogen when administered intradermally in BALB/c mice and side-by-side compared with the intramuscular immunization route. The results demonstrate that ID vaccination is well tolerated and triggered a significant magnitude of humoral antibody responses as similar to IM vaccination. Additionally, the ID immunization resulted in higher production of IFN-γ and IL-2 suggesting superior cellular response as compared to IM route. Overall, our data indicates immunization through ID route provides a promising alternative approach for the development of self-administrative SARS-CoV-2 vaccine candidates.

SARS-CoV-2 virus infection in humans has resulted in the COVID-19 pandemic which affected more than 87 million people all over the world and approximately 3.1 million deaths till March 2021 [1]. Since the early occurrence on December 31, 2019, in Wuhan provinces, China, the virus has now been spread over 220 countries, and has a massive impact on healthcare, livelihood, and socio-economic condition [2]. The SARS-CoV-2 infection can range from asymptomatic, mild to severe form based on the clinical signs and symptoms [3]. The most vulnerable and high-risk population is the old people (above 60 years), and people' with underlying health conditions [4]. In clinical cases, intensive-care requirement for SARS-CoV-2 infection is for the patients who can develop acute respiratory distress syndrome (ARDS), thus leading to severe respiratory distress and requirement of invasive mechanical ventilation and is one of the main reasons of COVID-19 associated deaths [5].

SARS-CoV-2 is an enveloped, RNA virus that belongs to the family Coronaviridae and placed in the genus beta Coronaviruses which also include known Coronaviruses of humans and animals such as SARS-CoV, Middle East respiratory syndrome Coronavirus (MERS-CoV), HCoV-OC43, HCoV-HKU1 and bat Coronaviruses [6,7]. Even though, the SARS-CoV-2 has a genetic similarity with the other Coronaviruses, there is a striking difference between the

Abbreviations: RBD, Receptor binding protein; ID, Intradermal; IM, Intramuscular; hACE2, human-angiotensin-converting enzyme 2; COVID-19, Coronavirus disease 2019; SARS-CoV-2, Severe acute respiratory syndrome-related Coronavirus 2; MERS-CoV, Middle East respiratory syndrome Coronavirus; SARS-CoV, Severe acute respiratory syndrome-related Coronavirus; S protein, Spike protein; TMB, 3,3',5,5'-Tetramethylbenzidine; ELISA, Enzyme-Linked Immunosorbent Assay.

* Corresponding author. Translational Health Science and Technology Institute (THSTI), NCR Biotech Science Cluster, 3rd Milestone, Gurgaon Expressway, PO box #04, Faridabad, 121001 Haryana, India.
E-mail address: sweety.samal@thsti.res.in (S. Samal).

[1] Equal contribution.

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virus–host interaction, the disease pathobiology, and transmissibility. The SARS-CoV-2 virus infection exhibits a higher or similar basic reproductive number (R_0) as compared to SARS-CoV [8–10] MERS, or influenza that results in higher transmissibility and spread of the virus [11,12]. One of the fundamental complications of SARS-CoV-2 is that it is a RNA virus, hence it is highly susceptible to rapid mutation and evolution of new variants. A new variant of SARS-CoV-2, Variant VOC 202012/01, has already emerged in UK showing a more rapid transmission rate among humans than that of the parent strain [13]. Vaccination has been initiated with approved vaccine candidates from Oxford-AstraZeneca's covid-19 vaccine, Pfizer and BioNTech mRNA, and Moderna mRNA vaccine globally, while about 321 other vaccine candidates are in various stages of development [8,14]. However, it will be highly beneficial to be prepared for next-generation vaccine development that can prevent virus escape and will require less human contact and resources. The above-mentioned vaccine candidates have adopted the intramuscular route which is a common route for injectable vaccines.

ID administration of the vaccine has the advantage of the usage of less volume of antigen and could present the antigen directly to antigen-presenting cells like dendritic cells for further processing and presentation and thus can trigger both the B cell and T cell arm for protection [15,16]. The route of administration of vaccines plays an important role in reaching out to mass population. In the present study, we have evaluated the immunogenicity of SARS-CoV-2 RBD peptide-based immunogen through ID route and further compared it with IM route of administration. Our results revealed ID route induced strong humoral response in the prime-boost approach and the antibody response is skewed towards IgG1 as compared to the IM route. Both the immunization routes also showed neutralization against the SARS-CoV-2 pseudotyped and wild type viruses. Furthermore, immunized mice through ID route showed significant T cell response in draining lymph nodes. Altogether, we have shown as a proof of concept, the ID route could also work as an ideal route of vaccine delivery and there is a scope of developing a self immunizable needle-free vaccine candidate against SARS-CoV-2 virus.

1. Material and methods

1.1. Animal immunization

The sequence of SARS-CoV-2 used for designing the peptides was based on the amino acid sequence of SARS-CoV-2 isolate Wuhan-Hu-1, GenBank: MN908947.3. A 20-mer peptide was selected from the receptor binding motif (RBM) region of RBD as peptide immunogen. In our recent study, we have shown that this 20-mer RBD peptide immunogen in combination with Freund's' adjuvant could induce potent antibody responses in BALB/c mice when immunized intra muscularly [17]. It was shown that the binding to this region is known to directly interfere with the binding of RBD with hACE2 [18]. The peptide was dissolved in phosphate-buffered saline (PBS) at pH 7.4 to a final concentration of 1–4 mg/ml. All animal studies were conducted in compliance with CPCSEA and were approved by the THSTI's Institutional Animal Ethics Committee (IAEC) having approval number IAEC/THSTI/104. 20 inbred adult male or female BALB/c mice of 6–8 weeks old were randomly allocated into four groups of five animals each (n = 5). BALB/c mice were administered with 40 μg of RBD peptide prepared in 1:1 with Addavax™ (adjuvant) via both intradermal route (n = 5), intramuscular route (n = 5), and their respective sham controls (n = 5). Blood collected from mice at indicated time points were incubated at 37 °C for 4 h and centrifuged to harvest the serum. The serum samples were heat inactivated at 56 °C for 1 h and stored at −20 °C for future use.

1.2. ELISA (Enzyme-linked immunosorbent assay)

Serum humoral antibodies generated against RBD peptide and spike protein were assessed by ELISA as described previously [19].

1.3. Lymph node and spleen single cell preparation

Single cell suspension of lymph nodes and spleen were prepared by mincing with frosted slides. Minced cells were pelleted by centrifuging at 1200 rpm for 5 min. Lymph node cells were resuspended in fresh complete media and processed further for experiment. Spleen cells were washed once with 1X PBS, and then lysed with 2.0 ml of RBC lysis buffer for 3 min at room temperature. After RBC lysis, spleen samples were washed twice with complete media and finally resuspended in complete media for further experiment.

1.4. Antigen stimulation and multicolor fluorescence staining

Briefly, the cells were stimulated with antigen (10 μg/ml) for 5 h at 37 °C in presence of brefeldin A. After incubation, cells were processed for cell surface and intracellular staining. Cells were fixed with 1% PFA and acquired in flow-cytometer. Detailed methodology is provided in supplementary information.

1.5. Virus neutralization

Pseudovirus based neutralization and the classical plaque-based neutralization assay using SARS-Related Coronavirus 2, Isolate USA–WA1/2020 virus was performed as per the standard protocol detailed in the supplementary information.

1.6. Cell–cell fusion assay

Cell–cell fusion assay was conducted as described before [20] with slight modification provided in supplementary information.

1.7. Statistical analysis

Statistical analyses were performed using the analysis software within the GraphPad Prism package 8. Two-way ANOVA test was used in ELISA. T cell data were presented as the means ± standard errors of the mean (SEM). The T cell assays were analyzed using one -way Anova, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 considered significant.

2. Results

2.1. Structural analysis of RBM peptide

The spike protein present on the SARS-CoV-2 virus surface is the major target of protective neutralizing antibodies and are present in the convalescent patient samples in response to SARS-CoV-2 infection. Neutralizing antibodies are elicited against different domains of the spike protein and particularly to the RBD [21,22]. Here, we have selected immunogenic peptide of 20 amino acids length that is a part of the RBM of RBD, the sequence of amino acids is shown in Fig. 1A. The neutralizing antibodies are known to bind to the RBM region both in SARS-CoV and SARS-CoV-2 [23]. Potent neutralizing antibodies such as S2E12 and S2H13 isolated from patient samples neutralize SARS-CoV-2 by targeting the RBM region of RBD [23,24].

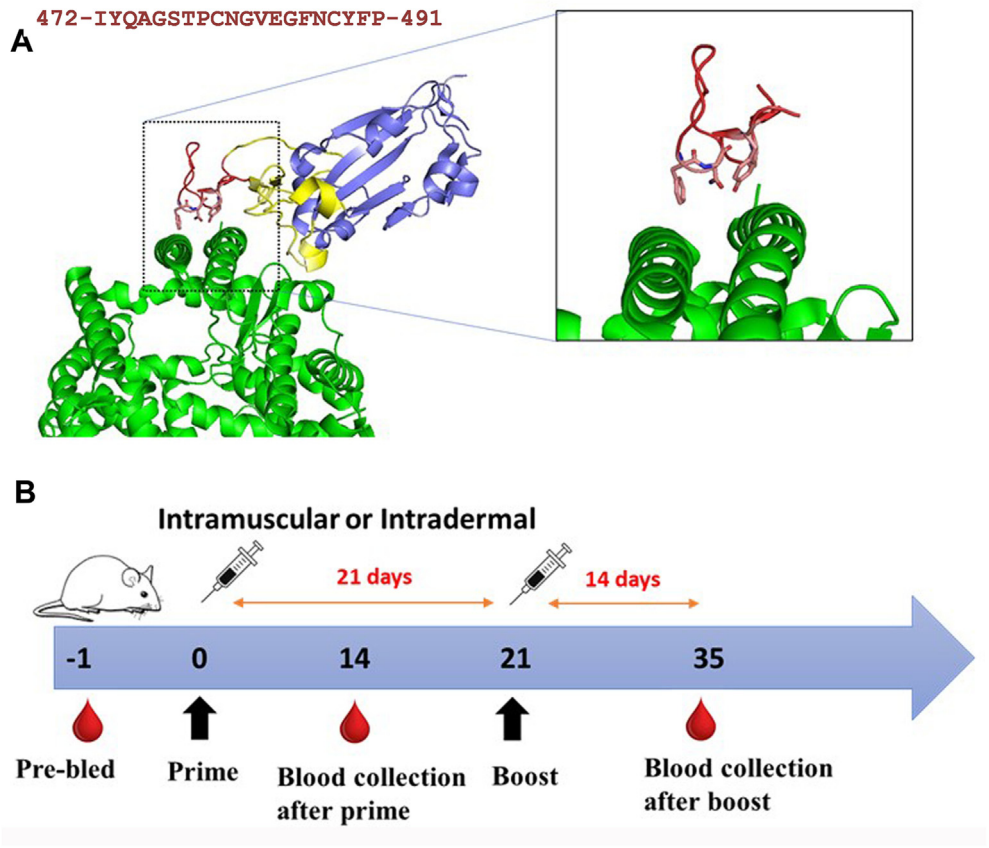

Fig. 1. Interaction of SARS-CoV-2 virus Spike-RBD protein with host ACE 2 protein and immunization of BALB/c mice. A. 3D modeled picture depicting the interaction of RBD (red, yellow and blue) domains of spike protein from SARS-CoV-2 virus with host ACE2 (green) protein. The RBM region is shown in yellow and the peptide region is shown in red. Rest of the RBD is shown in blue. The inset image showing the interacting face of ACE2 (green) with the selected peptide immunogen (red). B. BALB/C mice were primed and boosted at a gap of 3 weeks by intradermal and intramuscular routes. Blood was collected from mice by retro-orbital bleeding at indicated time points.

2.2. Immunization via both ID and IM route induces robust RBD protein specific humoral responses

Mice immunized with RBD peptide adjuvanted with Addavax™ in prime-boost regimen resulted in the elicitation of RBD-specific antibodies as measured by IgG ELISA. After 14 days of priming, mice immunized through ID route showed endpoint ELISA titer in between 1475 and 7250 dilutions to whole IgG, whereas sera from IM route immunized mice showed endpoint ELISA titer in between 1600 and 3625 dilutions (Fig. 2A and B). A single boost with RBD peptide immunogen at 28th day has resulted in a significant increase in humoral IgG response to RBD-whole soluble protein both in ID and IM route immunized group (Fig. 2A and B) ($P < 0.0001$, Two-way ANOVA). The whole IgG response was higher in IM group than ID group, which might be due to the injection site. In IM route, the antigen was injected directly to muscles which have high vascularity, that might have allowed fast mobilization of antigen to antigen presenting cells. We further profiled the type of IgG immune responses that have been generated in both ID and IM immunized routes and their comparative evaluation shows comparable humoral immune responses (Fig. 2D). As shown in Fig. 2C, IgG1 account for nearly half of the whole IgG responses in ID group, while in case of IM group induction of IgG1 in whole IgG was less as compared to ID group. Rest of the IgG responses in both the groups were other IgG isotypes directed (data not shown). Earlier we have shown that in case of IM group, IgG2 responses also makes significant portion in whole IgG [17]. It might be possible that IgG2 responses would be higher in IM group than the ID, and comparatively more Th2 polarization in case of IM immunization, which could also be reflected by the lower frequency of IFN-γ producing cells in IM group. In SARS-CoV-2 infection in humans, the lower respiratory tract is mainly protected by IgG1 antibodies [25]. The elicitation of IgG1 responses by either immunized routes, suggests ID route for immunization is an effective route to induce antigen-specific humoral responses.

2.3. The immunized serum binds to pre-fusion soluble spike protein and inhibits ACE2 mediated cell–cell fusion

Next, we determined the reactivity of the immunized sera to SARS-CoV-2 spike soluble protein. The sera from immunized mouse of each group were analyzed in ELISA against soluble prefusion spike protein. In the ID immunized group, the highest endpoint dilution reactivity to the soluble spike protein was found to be at ~30525 dilution and, in the IM immunized group the highest endpoint dilution was at ~51200 dilution (Fig. 3A). We further tested the ability of the sera to recognize the RBD and spike soluble protein through Western blot. The pooled sera after boosting from ID immunization was used as primary antibody in Western blot against soluble RBD and spike protein separated by 12% SDS-PAGE. Bands were recognized by mouse sera approximately at ~29 kDa to soluble RBD protein and at ~180 kDa to soluble spike protein (Fig. 3B) [22,25,26].

SARS-CoV-2 RBD protein interacts with hACE2 and thus facilitates virus entry into the host cell [27]. One of the characteristic

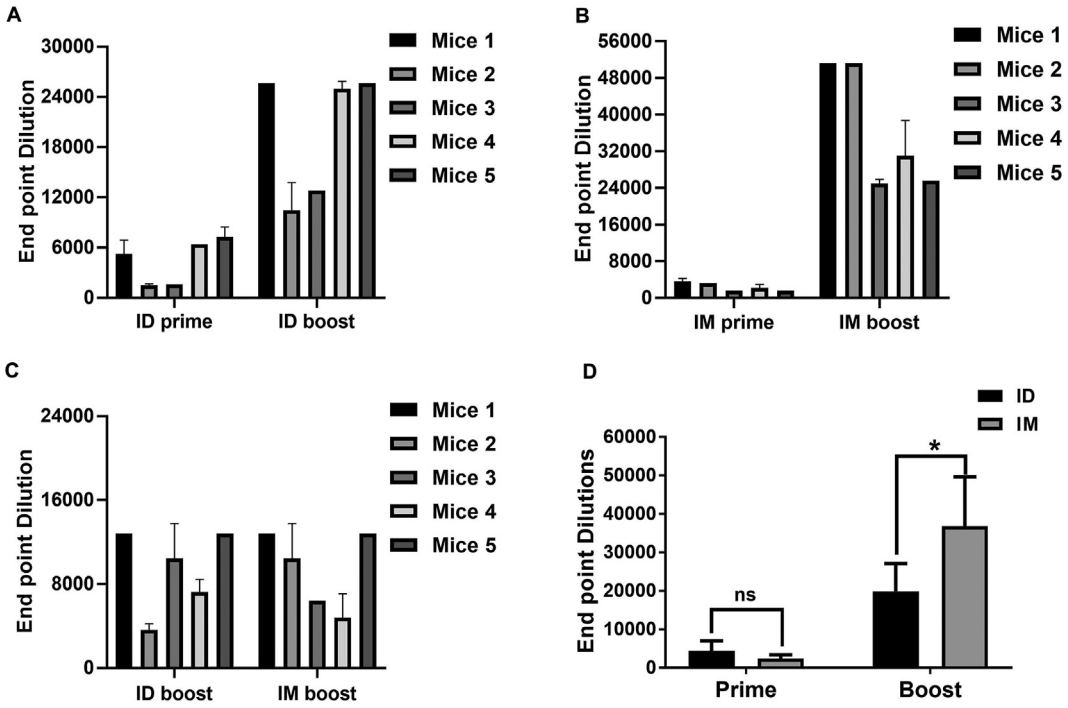

Fig. 2. Humoral responses generated with RBD peptide via intradermal and intramuscular route of immunization. A. Binding of serum IgG antibody titers of RBD via ID immunization route. B. Binding of serum IgG antibody titers of RBD via IM immunization route. C. Comparison of serum IgG1 and D. IgG antibody response generated after immunization with RBD peptide via ID and IM route of immunization. The plates were coated with RBD protein at concentration of 2 μg/ml. Values plotted are the mean endpoint titers in duplicate generated in five mice per group. Each bar represents single mean value of three repeated experiments. Statistical significance was determined using the one-way ANOVA test ($p < 0.05$).

features of the SARS-CoV-2 infected cells is to attach with the adjacent cells expressing receptors and thus forming syncytia. We established a cell-to-cell fusion assay which is a convenient method to measure the ability of sera to inhibit cell-to-cell fusion. As shown in Fig. 3C, in presence of control sera, the cell-to-cell fusion between 293T expressing full length spike protein and 293T stable expressing hACE2 is not inhibited, however in presence of immunized pooled sera from ID and IM group at 1: 100 dilution there is more than ~46.5 to 44.5% inhibition in cell-to-cell fusion was seen.

2.4. Route of administration influences induction of cellular immune responses

After 30 days post boosting, mice from both the ID and IM groups were dissected for spleen and draining lymph nodes. $CD4^+$ and $CD8^+$ T cell responses were measured in terms of IFN-γ and IL-2 cytokine production. The harvested cells from lymphoid organs were stimulated *in vitro* with the RBD peptide to measure the antigen specific cytokine responses in $CD4^+$ and $CD8^+$ T cells by intracellular staining (Fig. 4A and B).

In draining lymph nodes of ID immunized mice, CD4+ T cells producing IFN-γ were present in significantly higher frequency compared to that of IM, while the frequencies of IL-2 and IFN-γ + IL-2 producing cells were similar in both the routes (Fig. 4B.i). When we stimulated the cells *in vitro*, the frequency of the cytokine producing cells increased and was significantly higher in ID compared to that of IM group (Fig. 4B ii). In $CD8^+$ T cell population, at the resting stage the proportion of IL-2 and IFN-γ+IL-2 producing cells were significantly higher in ID group (Fig. 4C i), and upon *in vitro* antigen stimulation they have also maintained significantly higher frequency as compared to that of IM group (Fig. 4C ii).

Interestingly, in spleen we could not see any significant difference in the frequency of the cytokine producing $CD4^+$ (Fig. 4D) and $CD8^+$ (Fig. 4E) T cells among both the routes either at the resting stage (Fig. 4D and E) or upon *in vitro* antigen stimulation (Fig. 4D and E), except in ID group mice which showed significantly higher frequency of IFN-γ producing $CD4^+$ T cells than that of IM (Fig. 4D ii). However, here also, in both the routes we could see the increase in the frequency of cytokine-producing $CD4^+$ and $CD8^+$ T cells upon antigen stimulation (Fig. 4D ii & E. ii).

2.5. Serum antibodies neutralizes both spike pseudotyped virus and wild type virus

To test the immunized mice serum antibodies neutralizing ability, we performed S-glycoprotein pseudotyped in HIV plasmid backbone neutralization-based assay as described in material and methods section. The readout was percentage inhibition of relative luciferase units of S-glycoprotein pseudotyped viruses in presence of serum antibodies from pooled samples. As shown in Fig. 5A, the immunized pooled sera after the boost from both ID and IM have shown considerable neutralizing antibody titers, and in both ID and IM pooled sera at 1:320 dilution, more than 50% inhibition of pseudotyped viruses.

We further evaluated the neutralizing antibody responses of serum antibodies with wild type SARS-Related Coronavirus 2, Isolate USA–WA1/2020 virus by using plaque-based neutralization assay. In corroboration with pseudovirus-based neutralization assay, pooled sera from both ID and IM immunized mice after one boosting showed 50% inhibition in plaque formation as compared

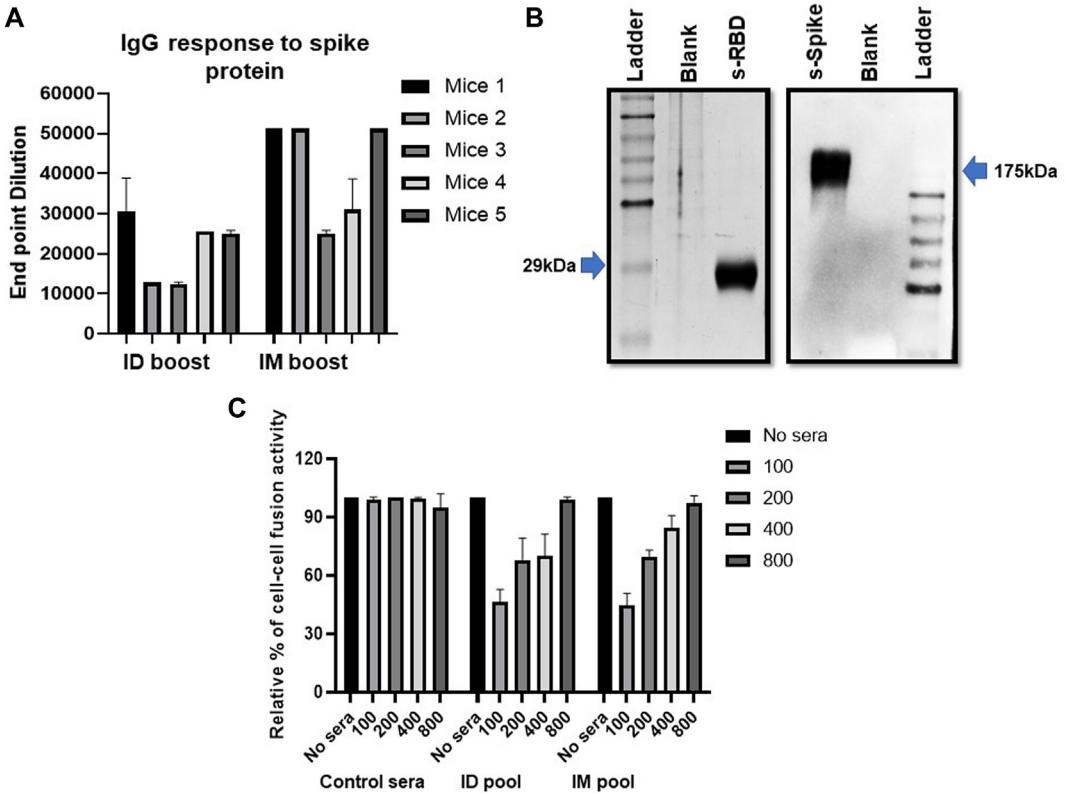

Fig. 3. Functional characterization of RBD immunized serum. A. Binding of serum IgG antibody to soluble spike protein after boost with RBD peptide via ID and IM route. B. Western blot analysis of RBD immunized serum reactivity to soluble spike and RBD protein separated on SDS-PAGE (4–12%) gels. The pooled sera from five mice immunized with RBD-pep (ID) group used as primary antibody in 1:500 dilution to detect soluble RBD protein (left panel) and soluble spike protein (right panel). C. Cell-to-cell fusion between 293T expressing full length spike protein and 293T stable expressing hACE2 is not inhibited in control sera, and in presence of immunized pooled sera from ID and IM group at 1: 100 dilution more than ~50% inhibition is observed in cell-to-cell fusion activity.

to wild type SARS-CoV-2 virus up to 1:160 dilutions (Fig. 5B) in Vero E6 cell. Both the assays suggest, the peptide immunization in both the routes was able to induce neutralizing antibody responses that can inhibit virus entry into host cells.

3. Discussion and conclusion

The ultimate goal of vaccination is to generate a robust and long-lasting protective immune response against a pathogen. Vaccine administration route which is less invasive and easily deliverable would have greater public accessibility. IM route dominates the existing vaccine delivery methods despite being more invasive in nature and requires much more care during administration. Although other routes of vaccine delivery were also shown to be either equally or more efficacious compared to that of IM route. ID is a promising route for vaccine delivery, particularly in the form of microneedle or microarray patches (MAP). Exploration of different routes of vaccine administration suggests that not only adjuvant but also the route of administration of identical antigens influences immune responses [28,29]. ID administration of influenza vaccine emerges as a promising alternative to conventional IM route [30]. So, far BCG and rabies are the only vaccines that is administered through the ID route. Also, ID route has several advantages over the IM route for being less invasive in nature, have a potential of greater public reach due to the feasibility of self-delivery, could be injectable at any site in the body, and more acceptable vaccination route for children and infants [16]. More interestingly, it could deliver in a targeted manner vaccine closer to the desired draining lymph node site to bring the maximum effect. Therefore, in this study we explored the efficacy of ID route in the generation of antigen specific immune responses as compared with IM route of immunization for a 20 mer RBD peptide of SARS-CoV-2 virus glycoprotein. Two doses of immunization using the only 20-mer RBD peptide along with the Addavax™ adjuvant resulted in the production of substantial humoral antibody responses against the soluble RBD, both by ID and IM route (Fig. 2), though there were differences in the magnitude of antibody responses. The high antibody responses generated by IM injective might be due to the fact the antigen is administered to muscle tissue with high vascularity which results in high mobilization and processing of antigen as compared to the ID route, in which the antigen is administered into the layer of subcutaneous fat which might resulted in delayed mobilization and processing of antigen [31,32]. The differences in antibody responses in ID vs IM route might be due to the difference in the site of injection which regulates antigen presentation and stimulation of adaptive immune responses. However,we have seen, through ID route even after priming there was the induction of antigen specific immune responses, as ID site is good at antigen presenting cells (APC), and the population of dendritic cells (DC) in the dermis is higher as compared to other sites which facilitate capture and local proliferation of DC and draining to local lymph nodes [33]. In addition, the antibody responses generated were directed towards IgG1, this is in corroboration with earlier findings with RBD vaccination with Addavax™ as an adjuvant results in higher induction of antibody responses [34]. We have also observed that the cellular T cell responses were quantitatively as well as qualitatively better for ID immunization. In ID route, the frequency of CD4+ IFN-γ+ and CD8+ IL-2+ cells in the draining lymph nodes were higher, which

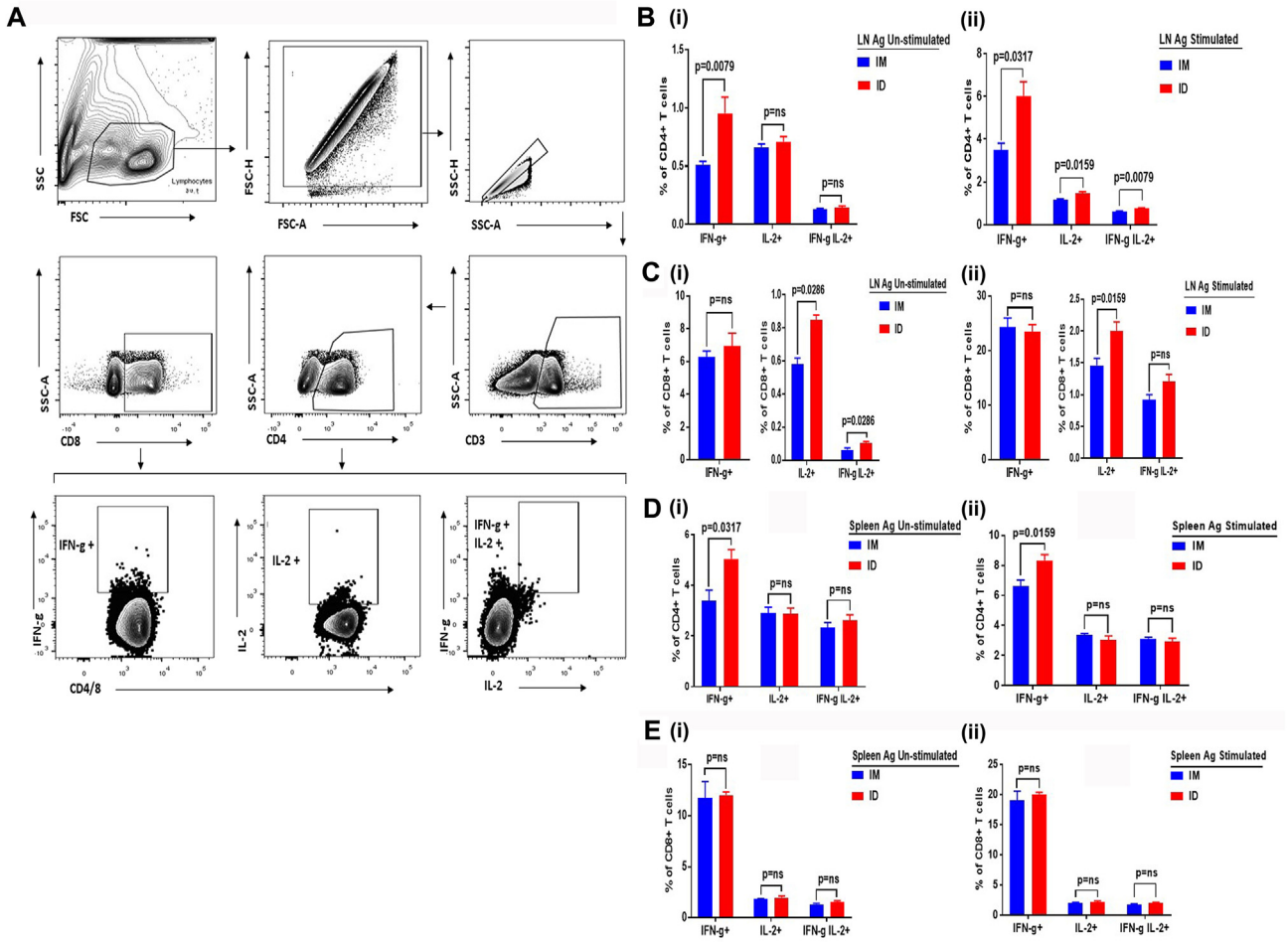

Fig. 4. Cellular responses as measured by flow cytometry: Representative contour plots showing the gating strategy for cytokines (IFN-g, IL-2) producing CD4+ and CD8+ T cells. A. Lymphocytes were gated based on SSC-A and FSC-A. Height and area parameter of FSC and SSC were used to gate on single cells. CD3 marker was used to gate the T cells, and further CD4 and CD8 markers were used to gate on CD4+ and CD8+ T cell population. Using controls, CD4+ and CD8+ T cells were gated onto respective cytokine producing cells. B-E. Comparison of ID v/s IM route for cytokine producing cells in draining lymph nodes and spleen. Frequency of lymph nodes resident CD4+ (B) and CD8+ (C) T cells producing cytokines at resting stage (i) and upon *in vitro* antigen stimulation (ii) in ID and IM route immunized mice group. Frequency of spleen resident CD4+ (D) and CD8+ (E) T cells producing cytokines at resting stage (i) and upon *in vitro* antigen stimulation (ii) in ID and IM route immunized mice group. Data are the mean ± SEM. The data were analyzed by non-parametric Mann–Whitney U-test. $P < 0.05$ is considered as significant. $N = 5$ mice per group. Data is from an experiment.

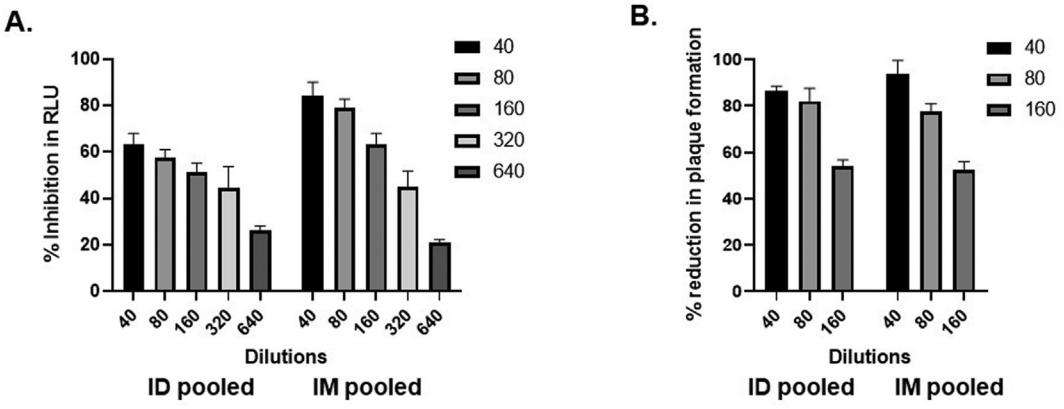

Fig. 5. Serum antibodies from ID and IM group neutralizes both spike pseudotyped virus and wild type viruses. A. Pseudoviruses prepared in pNL-4.3-luc HIV-1 back bone expressing SARS-CoV-2 spike protein were incubated with serum antibodies in duplicate for 1 h at 37 °C in 96-well flat-bottom plates. 293T-hACE2 cells (~25000 number) were added to the pseudovirus and serum mixture and incubated at 37 °C. After 48 hpi, cells were lysed and luciferase activity was measured by using the Britelite plus Luminescence Reporter Assay System. B. Plaque assays were performed using SARS-CoV-2 (USA–WA1/2020 isolate in Vero E6 cells. Sera were incubated with 50 PFU of viruses at 1:10 and 1:20 dilution for 1 h at RT and allowed to infect Vero E6 monolayer of cells seeded onto 24 well plate. Following 1 h of adsorption, the plate was washed with 2% DMEM and overlaid with 2% CMC prepared in DMEM. Forty-eight hpi the plates were fixed with 6% formalin for 4 h at room temperature and stained with 1% crystal violet to visualize the plaques.

depicts the quantitative difference of foreign antigen primed cells among the two routes. Moreover, the mean frequency of both IFN-γ + IL-2 producing CD4+ and CD8+ cells were higher in ID immunization, depicting the better-quality cells (Fig. 4). This difference in the T cell responses by the two routes of immunization could be attributed to the differential presence and diversity of DC among the two routes. While the difference among the two routes for T cell responses were noticeable in draining lymph nodes, similar responses were not observed for the spleen. The possible reasons could be the restricted involvement of spleen in the immune responses generated against the locally delivered antigen and also spleen lacks the afferent lymphatic vessel [35] which further restricts its involvement. However, increased frequency of IFN-γ producing CD4+ and CD8+ T cells in spleen upon *in vitro* cognate antigen stimulation depicted their engagement in the immune response. Interestingly, in spleen we observed significantly higher IFN-γ producing CD4+ T response in ID route which might account for the similar IgG1 responses for the two routes despite the fact that the whole IgG response was higher for IM after boost dose. And this observation might be useful for the designing of an effective vaccine candidate against SARS-CoV2 as it might have an impact on the outcome of route of vaccination [17]. One of the important activities of SARS family of viruses is the formation of receptor-mediated syncytia after the spike protein attaches to hACE2 receptor, thus activating the fusion process [36,37]. The cell-to-cell fusion of infected cells facilitates virus spread in between cells thus might be enhancing the pathogenesis. The sera from both the immunized group showed to block the viral entry at 1: 100 dilutions. In consistent to the above result, both pseudo virus and wild type virus-based neutralization assays have also shown to neutralize the virus during entry process.

In conclusion, in this study we have demonstrated that the ID immunization route is equivalently immunogenic as compared to traditional IM route of immunization. By using a short RBD peptide immunogen with Addavax™ adjuvant and minimal doses, high humoral and cellular responses can be achieved and the sera also have shown neutralizing potential. The present results are promising in the development of self-injectable vaccine patches for SARS-CoV-2 which require minimal human interventions and contact and thus will help in limiting the rapid spread of viruses.

Author contributions

NY, PV and RK carried out animal experiments; PV and RK carried out ELISA, GS carried out biochemical experiments; SS and RK carried out BSL3 virus experiments; SA carried out the soluble RBD and spike protein production; NY, PV, SA edited the manuscript; SS conceived the study, SS, SA and AA designed experiments, analyzed data, and SS wrote the original draft; SA and AA edited the manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2021.104843.

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