The promising application of nanostructured SBA-15 silica as oral adjuvant

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ABSTRACT

**Background:** Nanostructured mesoporous SBA-15 silica is a particle that due to its physicochemical properties shows great potential as an immune adjuvant. The present study evaluated the responsiveness of isogenic BALB/c mice orally immunized with Human Gama Globulin (HGG) encapsulated/adsorbed on SBA-15. **Results:** After proper oral immunization schedules, using SBA-15 as adjuvant, it was observed an increase in the serum IgG and IgA specific antibody titers and no influence of silica in the polarization to T\textsubscript{H}1 or T\textsubscript{H}2 immune responses. Flow cytometry analysis has shown that SBA-15 was efficient in promoting the recruitment of immunocompetent cells to the Peyer’s patches and to the mesenteric lymph nodes. **Conclusions:** SBA-15 silica is an effective, economically viable, stable and safe adjuvant/vehicle mainly for oral immunizations.
1. Introduction

Mesoporous SBA-15 silica is an inorganic material synthesized under acid conditions with ordered channels of uniform hexagonal nanostructured pores measuring approximately 10 nm in diameter. Described in 1998, these 30 µm particles of silicon oxide are able to interact with atoms, ions and molecules and due to their physicochemical properties they present potential as an adjuvant. Despite several studies focused on the use of this silica as a vehicle for other substances, it had not been tested for the ability of efficiently activate antigen presenting cells (APC), carry and release antigens [1-5].

The first study regarding the adjuvant effect of a nanostructured material in immunology was carried by our group through the use of SBA-15 [6]. Both isogenic BALB/c and heterogeneous genetically selected Low responder mice [7], immunized with distinct immunogens adsorbed/encapsulated in SBA-15, such as the *E. coli* Intimin-1β protein or *Micrurus ibiboboca* snake venom, a complex of distinct peptides and proteins, presented increase in specific antibody titers inducing equivalent or higher responsiveness when compared with immunizations using Incomplete Freund Adjuvant (IFA) or aluminum hydroxide - Al(OH)₃ [6]. Moreover, SBA-15 proved to be safe and non-toxic in addition to positively modulate the humoral immune responses of two Low responder mouse lines (LIII; LIVA), which after oral or intramuscular immunizations with bovine serum albumin (BSA), produced IgG titers as high as the High responder lines (HIII; HIVA) [6, 8]. These data indicate the high potential of SBA-15 in activating the immune system.

Since the natural routes of most infections are the mucosa, the administration of immunogens by this via can mimic the induction of natural immunizations. Oral
vaccinations can be particularly advantageous due to its easy administration and minimal side effects. Environmental factors, such as the harsh gastric environment, extremely acid and presenting highly proteases activity and the intestinal epithelium barriers may lead to antigen denaturation, with the destruction of essential epitopes, decreasing the capture of antigens by lamina propria (LP) APC and M cells at Peyer’s patches (PP), impairing significantly the immune responsiveness [9-16]. Thus, it is relevant the development of new mucosal adjuvants/vehicles able to effectively stimulate the immune system, modulating the response of low responder individuals, without any bias towards Th1 or Th2, besides being safe, economically viable and easily administrated [17-30]. Obviously, it is expected that vaccination process by the oral route result in the stimulation of the production of protective antibodies and in the induction of specific and long lasting immunologic memory.

Herein, it is presented the applicability of SBA-15 as an adjuvant to be used in oral immunizations. The high levels of specific seric antibodies and the increased numbers of immune cells found in the Gut Associated Lymphoid Tissue (GALT) after oral immunizations with Human Gamma Globulin (HGG), support our hypothesis that the incorporation of antigens to this mesoporous silica protects the main epitopes against the acid pH and enzymatic degradation, allowing its capture by APC at PP and LP, the stimulation of the draining mesenteric lymph nodes (MLN) and the enhancement of the immune response through the efficacious presentation of preserved epitopes.

2. Materials and methods

2.1 Animals
Female 8 to 12 week-old isogenic BALB/c mice, supplied by Butantan Institute animal facilities, were used to evaluate the adjuvant effect of SBA-15 silica toward oral immunizations. All animal experiments were conducted according to protocols approved by the Animal Use and Care Ethics Committees from the Institute of Biomedical Science of the University of São Paulo and from the Butantan Institute, and under ethical conditions according to the international rules of animal care by the International Animal Welfare Recommendations [31].

2.2 SBA-15 silica synthesis

Mesoporous SBA-15 silica samples were synthesized by using a poly-(ethylene oxide)-poly-(propylene oxide)-poly-(ethylene oxide) triblock copolymer (Pluronic P123, EO20PO70EO20, \( M_w = 5800 \) - BASF Chemical Co., Mount Olive, NJ, USA) acting as a micellar template. The source of silica for polymerization was the tetraethyl orthosilicate (TEOS), acquired from Fluka/Sigma Chemical Co. (Milwaukee, WI, USA). Hydrochloric acid was purchased from Fisher Scientific Co. (Pittsburgh, PA, USA).

2.3 Incorporation of Human Gamma Globulin in SBA-15 silica

The encapsulation and/or adsorption of HGG (Sigma-Aldrich Biotechnology Co., St. Louis, MO, USA) in SBA-15 were determined by \textit{in vitro} tests. Briefly, HGG (1000 µg/mL) was added v/v in different proportions (1:5, 1:10, 1:25 and 1:30) to SBA-15 diluted in phosphate buffer saline (PBS) pH 7.4, in a final volume of 1 mL,
and maintained at 4 °C for 24 hours. The non-encapsulated/adsorbed protein was removed by centrifugation at 400 x g for 5 minutes and the HGG concentration (µg/mL) in the supernatant was determined by the bicinchoninic acid assay (BCA kit - Thermo Scientific Pierce, Rockford, IL, USA) and spectrophotometric measurement at 562 nm.

2.4 Immunizations

Animals (n= 5 per group) received, by gavage, 10 µg of HGG adsorbed on SBA-15 in a final volume of 0.2 mL PBS. HGG was mixed in a 1:25 ratio antigen: SBA-15, v/v, (10 µg and 250 µg of silica, respectively). The mixtures were kept at 4 °C for 24 hours before immunizations. A booster was administered 45 days after the first immunization. Individual blood (retro orbital plexus bleeds) and pooled faecal samples were periodically collected for specific anti-HGG titrations by ELISA.

For morphological, histological and flow cytometry analysis, groups of BALB/c mice were immunized and euthanized 1, 3 and 7 days after primary administration. Small intestine was removed, washed with PBS and processed for light microscopy of PP. Histological slides were stained with hematoxylin-eosin.

2.5 Fecal pellet extract collection

Fresh fecal pellets, from orally immunized mice were collected, weighed and 5 mL of the inhibitory solution, i. e., 1mM phenylmethanesulfonylfluoride (PMSF) (Boehringer Mannheim, Indianapolis, USA) BSA 1% in PBS per 1 gram of fecal pellet, was added. After 15 minutes, samples were homogenized and centrifuged at 20,000 x g
for 10 minutes at 4 °C. The supernatants were removed and stored at -80 °C for ELISA assays.

2.6 Specific anti-HGG titrations

To determine specific anti-HGG antibody titers by ELISA [32], 96-well microplates with high binding properties (Maxisorp Nunc International, Rochester, NY, USA) were coated with 1 µg/mL of the corresponding antigen diluted in carbonate-bicarbonate buffer pH 9.6 (0.05 M Na₂CO₃ and 0.05 M NaHCO₃) for 16 hours at 4 °C. After incubation, plates were washed with PBS containing 0.05% Tween (PBS-T) and blocked with 0.5 % gelatin in PBS-T for 2 hours at 37 °C. Samples were serially diluted in PBS-T/gelatin 0.5%, and incubated for 1 hour at 37 °C to quantify IgG and its isotypes and for 18 hours to serum and secreted IgA determinations. Microplates were washed and 100 µL of peroxidase-labeled anti-IgG (diluted 1:2,000), anti-IgG1 (1:1,000), anti-IgG2a (1:1,000) or anti-IgA (1:500) (Promega Co., Madison, WI, USA) was added and the plates incubated for 1 hour at room temperature. After wash, 100 µL of buffer substrate (20 mg o-phenylenediamine, OPD, Sigma-Aldrich Biotechnology Co., St. Louis, MO, USA) diluted in 40 mL citrate-phosphate buffer (pH 5.0, 0.1 M citric acid, sodium phosphate, biphasic 0.2 M/H₂O₂ 0.3%) was added and incubated in the darkroom. Reaction was stopped with 50 µL/well of citric acid 0.2 M and read at λ450 nm (Multiskan - Labsystems, Helsinki, Finland). Individual antibody titers were expressed as $\frac{X}{\text{SD}}$ [log₂], calculated as the reciprocal serum dilution giving an absorbance of 20% of the plateau value.

2.7 Preparation of cell suspensions
Payer patches (6 per animal) and MLN were placed in Petri dishes containing RPMI 1640 medium (Cultilab Ltda., São Paulo, SP, Brazil) supplemented with 0.2% of fetal bovine serum (SBF, Cultilab Ltda, Brazil) and teased gently against sterile nylon wool. Cell suspensions from 5 animals were pooled and washed three times with supplemented medium, followed by centrifugation at 600 x g for 8 minutes at 4 °C. The number of cells was determined in a Neubauer chamber and viability of cell preparations routinely exceeded 90%, as judged by Trypan Blue staining.

2.8 Flow cytometry analysis

Pooled cells obtained from 5 BALB/c mice, either of the control or of the experimental groups, were analyzed using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Cell staining (2 x 10^5 cells/mL) was performed using monoclonal antibodies (BD PharMingen, San Diego, CA, USA) at appropriate concentrations as determined by titration: fluorescein isothiocyanate (FITC)-conjugated mouse IgG2a,κ anti-rat CD4 (clone RM4-5), mouse IgG1κ anti-rat CD45R/B220 (clone RA3-6B2), mouse IgG2b,κ anti-rat CD11b (clone M1/70), mouse IgG1,λ2 anti-armenian hamster CD11c (clone HL3) or R-Phycoerythrin (PE)-conjugated mouse IgG2a,κ anti-rat CD8α (clone 53-6.7). To reduce nonspecific antibody binding, Fc block hamster anti-mouse was included in cell surface staining. Data were analyzed for at least 10,000 events with BD Cell Quest® version 3.3 (Becton Dickinson, San Jose, CA, USA) and the results were expressed as mean values ± standard deviation of the numbers of positively stained cells in the population gated in at least two independent experiments. The number of each cell population per PP and MLN were determined by multiplying
its respective frequency among PP and MLN by the total number of cells per organ estimated in a Neubauer chamber [33].

2.9 Statistical analysis

Statistical significance was determined by unpaired t-test and set at $p < 0.05$ using GraphPad Prism 4.0 software (La Jolla, CA, USA).

3. Results

3.1. Incorporation of Human Gamma Globulin by the nanostructured SBA-15

Previous data through incorporation assays showed that proteins such as Intimin-1β and BSA are easily encapsulated into SBA-15 [8]. The in vitro incorporation analysis of the 150 kDa HGG in SBA-15 demonstrated that 99.5% of the HGG was encapsulated/adsorbed in silica at the ratio 1:25 (protein: SBA-15) – Table 1.

3.2. Specific antibody production after oral immunizations

Ten days after the booster, BALB/c mice orally immunized with HGG in SBA-15 produced s-IgA anti-HGG titers 2.5 times higher than those receiving HGG (Figure 1A). Serum IgA in the HGG:SBA-15 group were ~ 6.5 times higher at day 7 post-immunization (p.i.) when compared with mice immunized without this adjuvant; no difference was observed after the booster (Figure 1B). At the 7th day after the primary administration, serum IgG anti-HGG titers in HGG:SBA-15 immunized mice showed a
4–fold increase when compared with that produced by animals immunized without silica. This difference remained after the booster and the antibody levels in the HGG:SBA-15 group were 3 times higher than the HGG group (Figure 1C).

Based on previous results, it was evaluated the production of IgG isotypes during primary and secondary responses of orally immunized BALB/c mice with HGG or HGG:SBA-15. Results showed the predominant production of IgG2a in both groups (Figure 1D).

3.3. Morphological and histological analysis of GALT after oral immunizations

There were no morphological chances at PP or MLN on HGG or HGG:SBA-15 groups (data not shown). However, there was an increase in the numbers of cells per PP and MLN in HGG:SBA-15 immunized mice at 1, 3 and 7 days p.i.. The number of total cells was two times higher in HGG:SBA-15 mice at days 1 and 3 p.i. in the lymph nodes in comparison with HGG immunized mice in this period (Table 2). No changes were observed in the numbers of cells in non-immunized mice or in animals that received SBA-15 only (data not shown).

3.4. Cell profile at GALT after oral immunizations

Flow cytometry measures were done through the qualitative and quantitative analysis of cell types found on PP and MLN from BALB/c mice orally immunized with HGG encapsulated/adsorbed or not in silica. In the initial immune response it was observed a tendency for higher number of CD4⁺, CD8⁺, CD11b⁺, CD11c⁺, B220⁺ cells
per PP in the HGG:SBA–15 group, but only to CD11c+ cells were significantly different between groups at day 3 post-immunization (p<0.05) (Figures 2A – 2E).

At the 3rd and 7th days MLN from mice that received HGG:SBA–15 there was an increasing number of CD4+, CD8+, CD11b+, CD11c+, B220+ cells compared to the HGG group; however just in the 3rd day these differences were significant (p<0.05), except for CD11c+ cells (Figures 3A – 3E).

Moreover, analysis showed the presence of a subset of cells at PP and MLN with a low surface expression of B220+ marker, especially at day 7 p.i. as shown in Figure 4. This subpopulation may correspond to B lymphocytes at diverse stages of activation and a higher frequency of B220LOW subpopulation (28 %) in the HGG:SBA-15 immunized mice than in those receiving HGG (17 %), especially at PP level (Figure 4B). In contrast, cells from the MLN had a reduced population of B220LOW cells (7 %) in animals immunized with silica, in which the preponderant population was B220HIGH (26%) (Figure 4D).

4. Discussion

Nature is definitively the major vaccination agent, the most important and efficacious memory promoter. Thus, it could be assumed and kept in mind that a specific vaccine and/or vaccination schedules, besides its advantages in providing immunity, exerts selective pressures on pathogenicity.

The recognition processes hosts–pathogens occur inside and/or outside the cells, monitoring the molecular interactions. These complex relations guide the dynamic changes during ontogenesis and the maintenance of the conservative structures and mechanisms with highly adaptive values. Experimental data suggest the participation of
the APC in increasing the epitopic recognition, leading to an efficient induction of T and B lymphocytes memory and the prevention of clonal deletion and anergy, quantitative characteristics that are regulated independently by polygenes. Ineffective vaccination schedules, from the qualitative and quantitative viewpoint would act as selective agents for many characteristics of a pathogen or analogous microorganisms; and the quantitative traits such as virulence, toxicity, infectivity degrees, and repertory of receptors, phenotypes that are under modulation by the immune system, could negatively modify the natural history of the relationships between microorganisms and host species.

Considering that the mucosa is the natural locus of sensitization to a massive variety of antigens, the effectiveness of oral vaccinations could reduce the impact of environmental factors, diminish the entropy production, and modulate the virulent phenotypes of a given pathogen. Moreover, among these factors that act on the capacity and on the intensity of the immuneresponsiveness plus memory induction to pathogens, adjuvant plays important roles, mainly if non $T_{H1}$ or $T_{H2}$ selective inducers, a characteristic of the SBA-15.

Until now, few orally administrated vaccines were licensed for human use, such as those against poliomyelitis and rotavirus, both being highly immunogenic attenuated vaccines [11, 13, 14, 34]. The development of mucosal adjuvants or carrier systems is imperative to protect antigens from degradation within the gastro-intestinal tract, allowing them to reach immunological sites. In order to develop orally administered vaccines, new delivery systems have been tested aiming to sustain the immunogenicity of antigens and facilitate the capture of the immunogens by specialized cells; the effective stimulation of the APC will ascertain the positive modulation of antigen-specific B and T lymphocytes and ensure a long-term and systemic immunity [9-20, 25,
Considering the numerous benefits of immunizations by natural routes, it is of importance to think about the development of adjuvants that assist in maintaining the stability of antigens, especially considering developing countries where funds for health treatment and prevention are scarce. Many of these nations have a shortage of trained professionals, difficulties in storing and transporting vaccines, as well as the high costs involving the purchase of materials such as syringes and needles. Previous studies demonstrated the effectiveness of SBA-15 as adjuvant and here the proposal of using this nanostructure in oral immunization is presented [7, 8].

The incorporation analysis showed that the HGG was efficiently encapsulated and retained in the pores of SBA-15 silica that improved the specific immune response against such antigens (Table 1).

Another remarkable aspect to be considered is that particles with sizes between 2 to 5 µm are easily captured by the M cells at PP, while particles with 2 µm or less and the ones larger than 10 µm are quickly drained to the MLN [9, 10]. SBA-15 silica particles have approximately 30 µm; although not captured by M cells, they could be captured by DC located at the lamina propria, being an efficient carrier system, allowing the capture of antigens by the dendritic cells from lamina propria and MLN. When drained to these organs, antigens encapsulated/adsorbed to SBA-15 can be protected and slowly released, stimulating the development of local immune responses. Figure 1 shows increased levels of specific serum and secretory antibodies indicating that this silica is effective in inducing a systemic and locally produced antibody response without interfering in the polarization to a certain class of T\(_H\) lymphocytes. The differentiation of T\(_H\) lymphocytes in distinct subsets of effectors cells depends on cytokines, nature of antigens, infection route and activation of innate immune cells as well as the use of adjuvants [22-30]. Regularly, the oral mucosal microenvironment
tends to induce a T\textsubscript{H2} response due to the predominant concentration of cytokines such as IL-4, IL-5 and IL-10, especially after infectious processes or immunizations with proteins. Although, this polarization may not be suitable in protecting against certain infections like those that predominantly require a T\textsubscript{H1}-type response. In the absence of infections, mucosal CD103\textsuperscript{+} DC produces IL-10 and TGF-β keeping the T\textsubscript{H3} profile and suppressing the T\textsubscript{H2} responses [35].

Moreover, commensal microorganisms and antigens from feeding constantly stimulate the immune system resulting in the production of antibodies, sometimes undetected, or leading to oral tolerance, which is essential to prevent the induction of immunity against pathogens [16]. IgA is considered the first line of immune protection at the mucosa. Secretory IgA uses a high-affinity binding system to neutralize microbial toxins and pathogens, and a low-affinity binding system to prevent commensal bacteria from breaching the mucosal surface, maintaining the balance of the intestinal flora, preventing pathogens from entering the mucosa and facilitating its capture by the APCs. Systemic IgA binds to several receptors expressed on granulocytes, monocytes, macrophages, eosinophils, follicular DC and hepatocytes, supporting the internalization of opsonized bacteria. It is believed that these receptors act in a non-inflammatory defense against intestinal bacteria [34, 36-38]. There are distinct subsets of DC at the small intestine and they are essential for beginning a mucosal immune response against pathogens, control oral tolerance as well as the activity of regulatory T cells, participating in the generation, maintenance and down-regulation of mucosal inflammation induced by allergies or chronic diseases [35-43].

Corroborating the higher titers of specific antibodies after HGG:SBA-15 administration, the overview of the FACS analysis data evidenced a tendency in the increasing numbers of immunocompetent cells in PP and MLN from mice that received
the antigen on silica (Figures 2 and 3), point toward the improvement of the immune response after the use of this nanoparticle as an oral adjuvant. This same efficiency of SBA-15 has been observed to recombinant Hepatitis B vaccine and *E. coli* Intimin administered by the oral route (manuscript in preparation). It can be hypothesized that fragments of HGG reaches the PP in concentrations enough to stimulate the B220\textsuperscript{HIGH} cells in a similar way that the HGG encapsulated/adsorbed in SBA-15 (Figure 4).

The global analyses of our results determines the promising use of SBA-15 silica as an adjuvant to oral immunizations and demonstrate that this particle acts on the physical protection of antigens, promoting the efficient activation of the immune system.
Acknowledgments

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Authors’ contributions

All authors have read and approved the final manuscript.

Conceived and designed the experiments: KS, OS

Performed the experiments: KS, LC, GT, JF

Analyzed the data: KS, LC, GT, EM, DT, MF, OS

Contributed with reagents/materials: DT, MF, OS

Wrote the paper: KS, DT, OS
5. References


8. Carvalho LV, Ruiz Rde C, Scaramuzzi K, Marengo EB, Matos JR, Tambourgi DV, Fantini MC, Sant'Anna OA: **Immunological parameters related to the**


Legends of the Figures

FIGURE 1. Antibody production by orally immunized BALB/c mice. [A] Secretory IgA (s-IgA) anti-HGG; [B] Serum IgA anti-HGG; [C] Serum IgG anti-HGG; [D] Serum IgG1 and IgG2a anti-HGG titers produced after immunization with HGG or HGG:SBA-15. Assays performed by ELISA at day 7 p.i. (7dPR) and 10 days after booster (10dSR). Results are expressed as $\bar{X} \pm SD$ [log$_2$]; group of animals immunized with HGG was used as reference for the unpaired Student $t$ test analysis, * $p < 0.05$, ** $p < 0.01$.

Figure 2. Phenotypic distribution of cells at Peyer´s patches from orally immunized BALB/c mice. Mice immunized with HGG or HGG:SBA-15. Number of cells x $10^5$: [A] CD4+, [B] CD8+, [C] B220+, [D] CD11b+ and [E] CD11c+ cells at days 1, 3 and 7 p.i. analyzed by FACS. Results of two independent experiments expressed as $\bar{X} \pm SD$; n= 5 per group per experiments; animals immunized with HGG were used as reference for the unpaired Student $t$ test analysis; * $p < 0.05$.

Figure 3. Phenotypic distribution of mesenteric lymph node cells from orally immunized BALB/c mice. Mice immunized with HGG or HGG: SBA-15. Total number (cells x $10^5$) of [A] CD4+, [B] CD8+, [C] B220+, [D] CD11b+ and [E] CD11c+ cells at days 1, 3 and 7 p.i. analyzed by FACS. Results of two independent experiments [pool of cells] expressed as $\bar{X} \pm SD$; n= 5 per group per experiment; animals immunized with HGG were used as reference for the unpaired Student $t$ test analysis; * $p < 0.05$. 
Figure 4. Analysis of the B220 cell surface marker expression. [A] and [B] Peyer’s patches; [C] and [D] mesenteric lymph node cells, from mice orally immunized with HGG and HGG:SBA-15 separated by B220 FITC intensity (FL1) and CD8α PE intensity (FL2). Mice euthanized at day 7 p.i. Results representative of two independent experiments expressed as percentage of positive stained cells.
Table 1 – Encapsulation/Adsorption of Human Gamma Globulin in SBA-15 silica

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<th>Ratio HGG:SBA-15</th>
<th>Encapsulated HGG (%)</th>
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<tr>
<td>HGG (1000 µg/mL)</td>
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<tr>
<td>1:5</td>
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<tr>
<td>1:10</td>
<td>89.3</td>
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Table 2 - Total number of cells at Peyer´s patches and mesenteric lymph nodes of BALB/c mice orally immunized with Human Gama Globulin.

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<th>Peyer’s patches</th>
<th>Mesenteric Lymph Nodes</th>
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<tr>
<td></td>
<td>Day 1</td>
<td>Day 3</td>
</tr>
<tr>
<td>HGG</td>
<td>6.5 ± 2</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>HGG:SBA-15</td>
<td>8 ± 3</td>
<td>8 ± 2</td>
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Peyer’s patches (6 per animal) and mesenteric lymph nodes removed at days 1, 3 and 7 p.i.; n= 5 animals per group per experiment; pool of cells (cells x 10⁶). Results of two independent experiments expressed as X ± SD; group of animals immunized with HGG was used as reference for the unpaired Student t test analysis, * p < 0.05, *** p < 0.001.
Figure 1
Figure 2