Short Vi-polysaccharide abrogates T-independent immune response and hyporesponsiveness elicited by long Vi-CRM_{197} conjugate vaccine

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Supplemental information

Reagents

Reagents used were as follows: hydrogen peroxide (H_2O_2), sodium chloride (NaCl), sodium phosphate monobasic (NaH_2PO_4), N-Hydroxysuccinimide (NHS), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC), sodium acetate (AcONa), phosphate buffer solution (PBS), 2-(N-morpholino)ethanesulfonic acid (MES), adipic acid dihydrazide (ADH) [Sigma Aldrich]; acetonitrile (CH_3CN) [LC-MS Chromasolv]. Native Vi polysaccharide (PS) (average size of 165 kDa) was produced as previously described (1). CRM_{197} was obtained from GSK Siena.

Method for making short chain Vi and characterization

Vi, freeze dried as the sodium salt, was solubilized in water and H_2O_2 was added to give a final concentration of 2.5 mg/mL Vi and 5% (wt/v) H_2O_2 in water. The mixture was heated at 80±0.5°C for 2h. The mixture was then injected into a Hiscreen Capto Q [GE Healthcare] column (4.7 mL of resin loading upto 100 mg of fVi mixture) equilibrated with buffer A and four populations of different average size were separated using a gradient step method. NaH_2PO_4 20 mM pH 7.2 and NaH_2PO_4 20 mM NaCl 1M pH 7.2 were used as buffer A and B respectively. Pools of increasing average size Vi were eluted at 25, 30, 37 and 45% of buffer B respectively. Each pool was desalted on a Sephadex G-25 column [GE Healthcare] equilibrated with water.

The average size of the Vi pools was determined by HPLC-SEC equipped with a TSK gel 3000 PWXL column and a TSK gel PWXL guard column (Tosoh Bioscience) (Fig. S1). Dextrans (5, 25, 50, 80, 150 kDa) were used as standards (Sigma Aldrich). The mobile phase was 0.1 M NaCl, 0.1 M NaH_2PO_4, 5% CH_3CN, pH 7.2, at the flow rate of 0.5 mL/min (isocratic method for 30 min).

HPAEC-PAD was used to measure Vi content (2), and micro BCA (using N-acetylglucosamine as standard) to confirm CHO groups formation. ^1H NMR was used to verify Vi identity and calculate O-acetylation level (2).

Synthesis of Vi-CRM_{197} conjugates

The carboxylic groups along the PS chain were first activated with EDAC and NHS to generate NHS active esters. Subsequently the activated PS were randomly linked to the carrier protein, previously derivatized with ADH as a spacer (Fig. S2).

With Vi pools of average size 9.5, 22.8 and 42.7 kDa, the following procedure was used for conjugate preparation. Short chain Vi was solubilized in100 mM MES pH 6 at a concentration of 50 mg/mL. NHS and then EDAC were added to produce EDAC/Vi repeating units at a molar ratio of 5 and NHS at a concentration of 0.33 M. The reaction was mixed at room temperature for 1h. CRM_{197}-ADH, prepared as previously described (1), was added to give Vi and protein concentrations each at 7.8 mg/mL (Vi to

protein w/w ratio of 1) in 20 mM MES pH 6. The solution was mixed at room temperature for 2h. For 82.0kDa-Vi, reaction conditions described above resulted in gel formation. Thus, the activation step with EDAC/NHS was performed with a reduced Vi concentration of 15 mg/mL, and 0.1 M NHS. For 165kDa-Vi, the PS concentration was further reduced to 4.2 mg/mL. The protein concentration in the conjugation step was 7.8 mg/mL with short chain Vi populations, while it was reduced to 1.7-3.5 mg/mL for 165kDa-Vi (Vi to CRM_{197} w/w ratio of 1 in all cases).

Conjugates were purified by size exclusion chromatography, eluting in PBS at 0.5 mL/min on a 1.6 cm x 60 cm Sephacryl 100 HR column [GE Healthcare].

Purified conjugates were characterized by HPAEC-PAD for total Vi content (2), micro BCA for total protein content, HPLC-SEC for determining average size distribution of the conjugate (TSK gel 3000 PWXL column) and to assess the amounts of free protein (fluorescence emission detection) and free saccharide (9.5kDa-Vi-CRM_{197} and 22.8kDa-Vi-CRM_{197}; refractive index detection). For conjugates prepared with 42.7kDa-Vi, 82.0kDa-Vi, as for 165kDa-Vi-CRM_{197}, free saccharide was estimated by HPAEC-PAD following separation on Capto Adhere resin (3, 4).

Immune response in adult mice

Mice were purchased from Charles River Laboratory and maintained in accordance with Novartis Vaccines Animal Care policies. All animal protocols were approved by the local animal ethical committee and by the Italian Minister of Health in accordance with Italian law (AEC N° 201309).

All studies used groups of 10 week old SPF female mice (N = 8 per group). Immunization was by subcutaneous injection of 200 µL. Antigens were diluted in physiologic saline solution without adjuvant.

In the first study, ten groups of CD1 mice were immunized with Vi-CRM_{197} conjugates made from Vi of different sizes and with the corresponding unconjugated Vi PS (Table 1). Eight micrograms of Vi was delivered on days 0 and 35. Blood was collected at days 0, 14, 35 and 49 for serologic assessment.

A subsequent study was performed to compare Vi antigens in wild type and T-cell deficient nude mice. Five groups of CD1 wild type and five groups of CD1 nude mice were immunized with Vi-CRM_{197} conjugates made from different size Vi and with a unconjugated 165kD-Vi PS. Eight micrograms of Vi were administered on days 0 and 28. Blood was collected at days 0, 14, 28 and 42 for serologic assessment.

Immune response in neonatal mice

Adult NMRI mice were purchased from Taconic (Skensved, Denmark) and allowed to adapt for 1 week before mating. They were kept in microisolator cages with free access to commercial food pellets and water, and housed under standardized conditions at ArcticLAS vivarium facility (Reykjavík, Iceland), with regulated daylight, humidity and temperature. Breeding cages were checked daily for new births, and pups kept with their mothers until weaning at 4 weeks of age. This study was carried out in accordance

with the Act No. 55/2013 on animal welfare and regulations 460/2017 on protection of animals used for scientific research. The protocol was approved by the Experimental Animal Committee of Iceland (license no. 2016-11-01).

Neonatal (1 week old) mice (8 mice/group) were immunized subcutaneously at base of tail with two doses of either 8.0 μg of 42.7kDa-fVi-CRM_{197} or 165kDa-Vi-CRM_{197} mixed with the adjuvant alum (0.48% aluminum hydroxide per 1 μg of protein/mouse), at a 3 weeks interval in 50 μL or 100 μL of saline, respectively. Two weeks after the second dose, the mice (now 6 weeks old) received a booster with 8.0 μg of unconjugated 42.7kDa-fVi, unconjugated 165kDa-Vi, the same conjugate used in the first two doses, or saline in 200 μL of saline. No alum was included into the formulation for the third injection.

Blood was obtained from the tail vein at various time points; serum was isolated and stored at −20°C. Spleens were removed and BM was collected for enumeration of Vi- and CRM_{197}-specific ASC and phenotyping of Vi-specific B cells.

ELISA

Vi-specific antibodies were measured by ELISA as previously described (5). 165kDa-Vi was used for ELISA plate coating (1 μg/mL in phosphate buffer).

ELISPOT

Vi- and CRM_{197}-specific ASC were enumerated by ELISPOT, as previously described (6-8) using 10 μg/mL Vi or CRM_{197} coated plates. The number of spots were counted by ELISPOT reader ImmunoSpot® S6 ULTIMATE using ImmunoSpot® SOFTWARE (Cellular Technology Limited (CTL) Europe, Bonn, Germany).

Flow Cytometry

Single-cell suspensions were prepared from spleen and BM, as previously described (6, 7, 9), where Vi-specific cell were identified by staining with fluorocrome-labeled Vi PS and antibodies (BD Biosciences): anti-B220 (clone RA3-6B2), AnnexinV and anti-Ki67 (clone B56) and analyzed using Navios cytometer (Beckman Coulter, Brea, CA, USA). The data generated were analyzed using Kaluza® analysis software (version 1.3, Beckman Coulter).

Statistical and graphical analysis

Analysis was performed using GraphPad Prism 6 or 7.03 (GraphPad Software, La Jolla, CA, USA). Mann-Whitney *U* test was used to compare two groups and Kruskal-Wallis analysis with post-hoc Dunn's test to compare multiple groups, Wilcoxon matched-pairs signed rank two-tailed test was used to compare results from the same group at different time points.

References:

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Supplemental figures

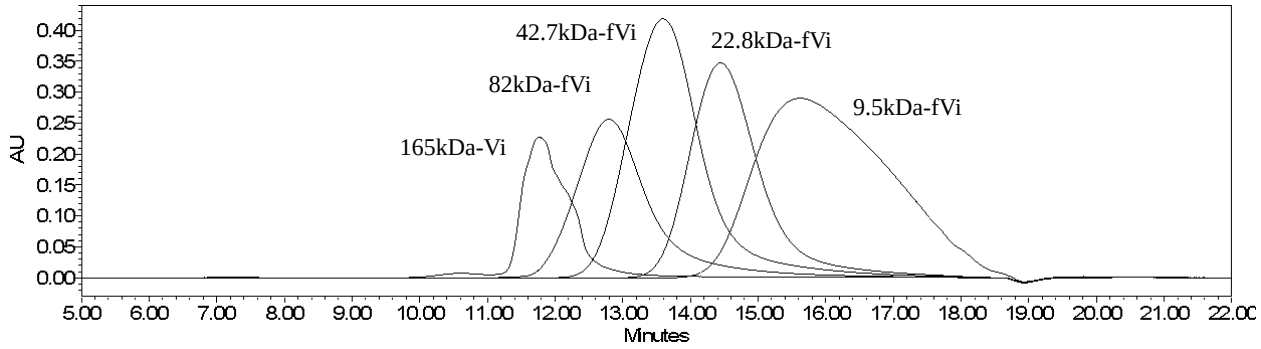

Fig. S1. Characterization by HPLC-SEC of Vi populations of different average sizes compared to long 165kDa-Vi. Detection at 214 nm, TSK gel G3000 PWXL column with TSK gel PWXL guard column, 0.1 M NaCl, 0.1 M NaH_2PO_4, 5% CH_3CN, pH 7.2 at a flow rate of 0.5 mL/min; average size calculated with respect to dextran standards, using refractive index detector.

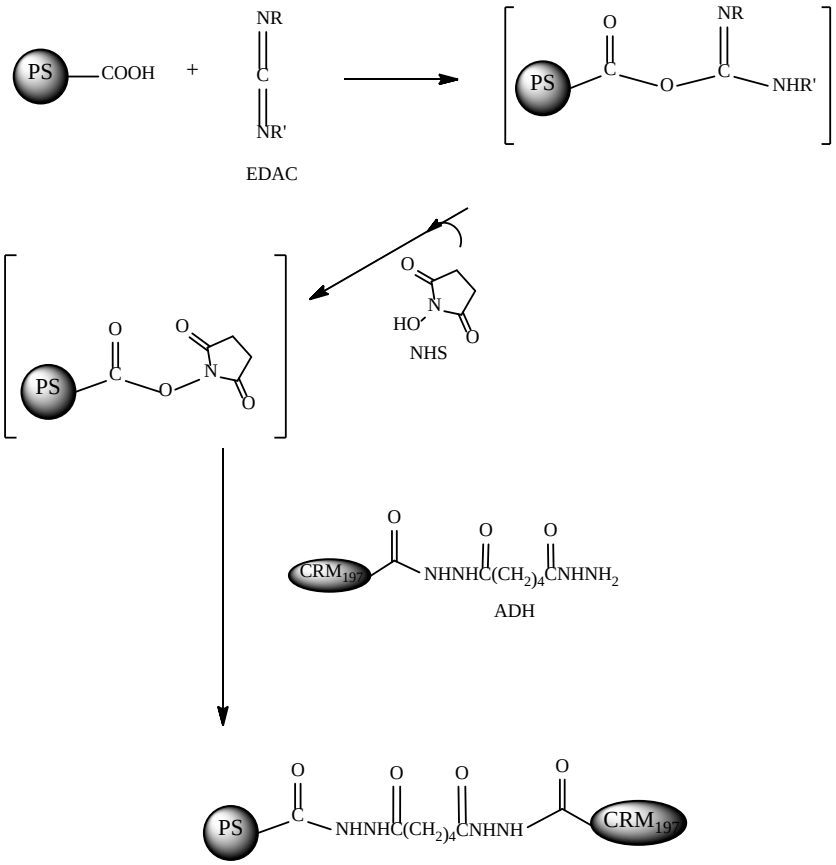

Fig. S2. Conjugation scheme applied to Vi for covalent linkage to the carrier protein CRM_{197}. Carboxylic groups of Vi are activated with carbodiimide (EDAC) and N-hydroxysuccinimide (NHS) in order to form a semi-stable amino reactive intermediate. This intermediate reacts with CRM_{197} previously derivatised with adipic acid dihydrazide (ADH) linkers.

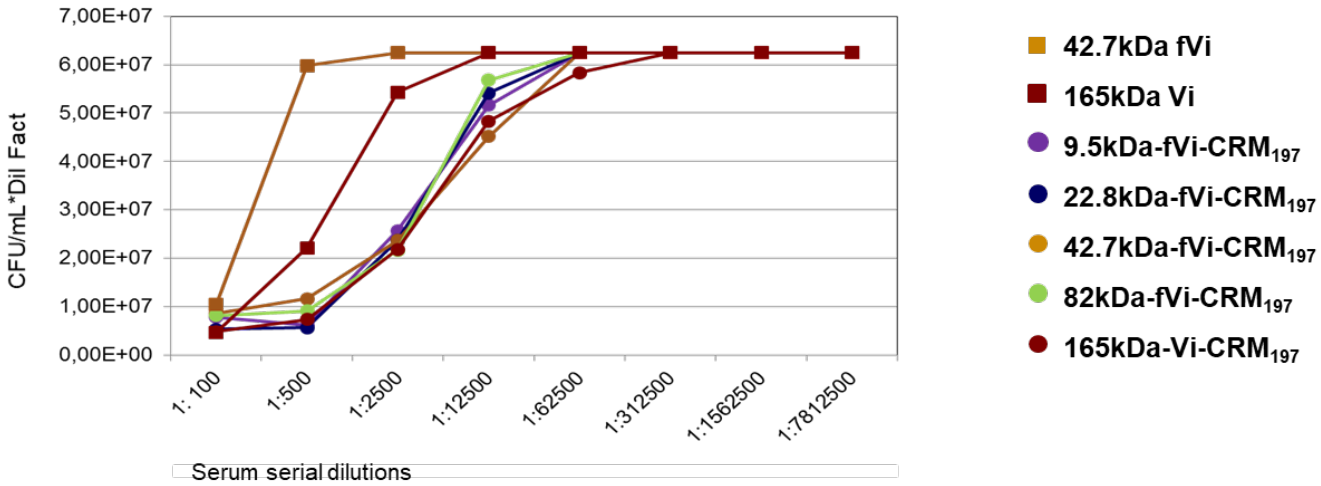

Fig. S3. **Serum bactericidal activity in mice immunized with Vi-CRM$_{197}$ conjugates containing Vi polysaccharide of different length.** Subcutaneous injections containing 8 µg of Vi polysaccharide were given at days 0 and 35. Pooled sera collected two weeks after the second injection were tested for bactericidal activity against a *Citrobacter freundii* strain expressing Vi. Sera from mice immunized with unconjugated 165kDa and 42.7kDa Vi were also tested for comparison.

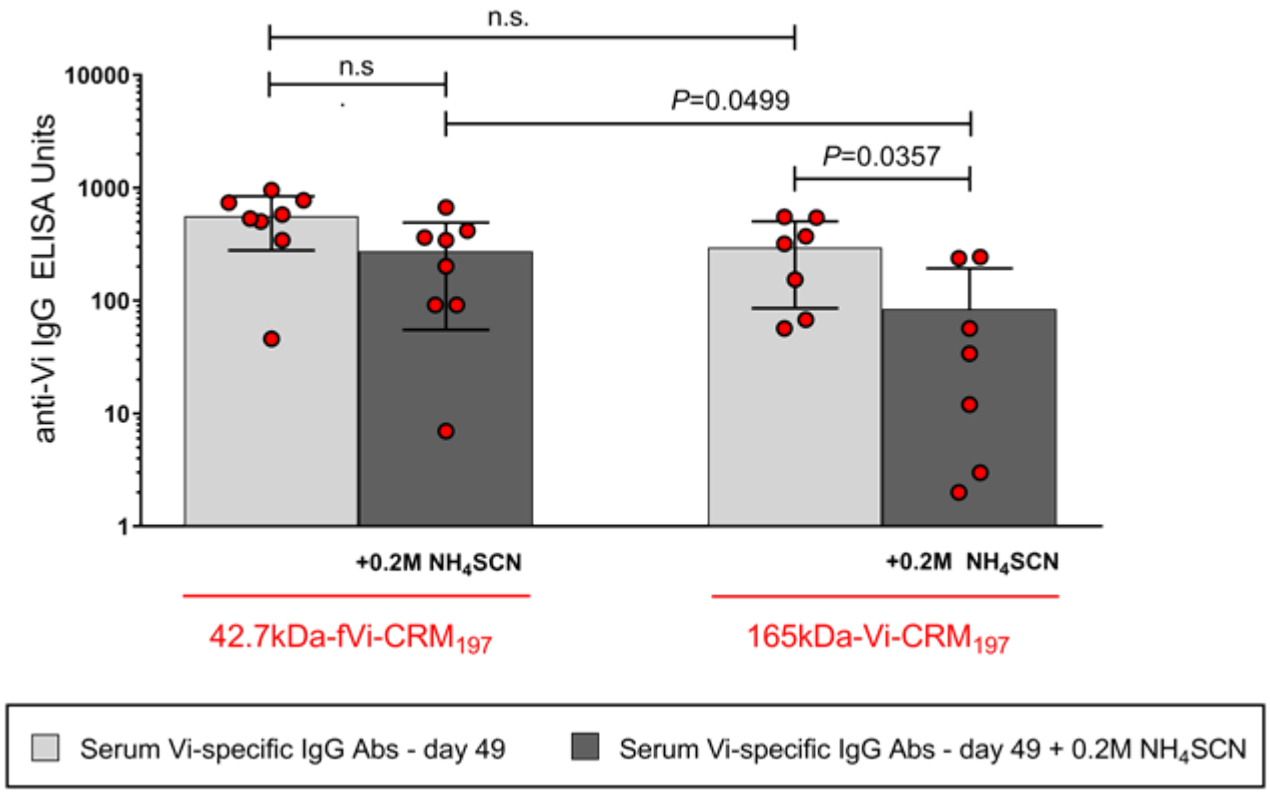

Fig. S4. Avidity of sera from mice boosted with the two conjugates was compared, by measuring anti-Vi IgG ELISA units in the presence of 0.2 M ammonium thiocyanate (NH_4SCN).

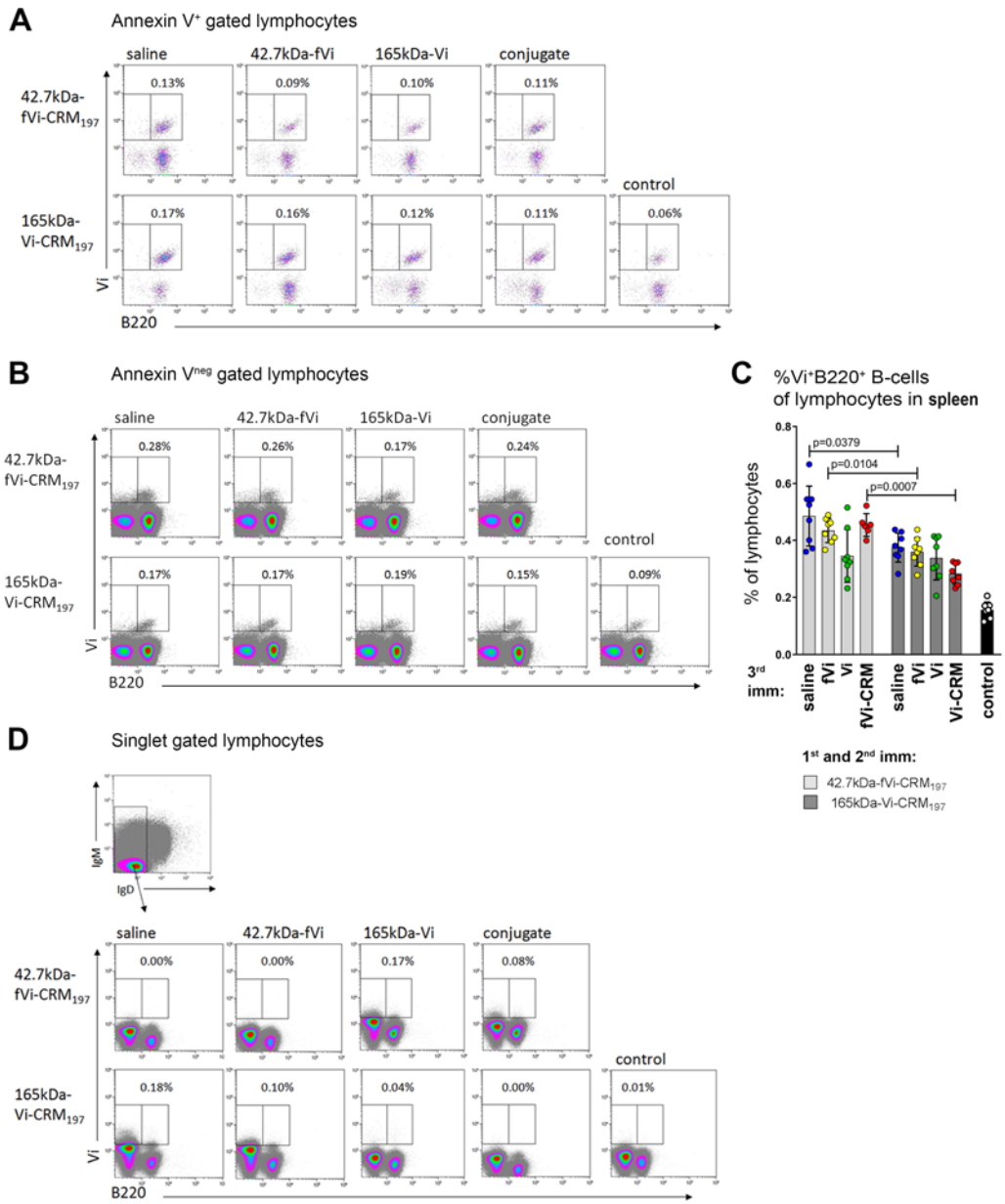

Fig. S5. Frequency of Vi-specific B220^{+} cells among lymphocytes and dotplots among Annexin V^{+} or Annexin V^{neg} cells in spleen and among IgM$^{+/neg}$IgD^{neg} in BM, 12h after the 3rd immunization. The mice were immunized with two doses of 42.7kDa-fVi-CRM$_{197}$ or 165kDa-Vi-CRM$_{197}$ with 3 weeks interval and 2 weeks after the second dose the mice received different boosters, either saline (blue circles), unconjugated 42.7kD-fVi (yellow circles), unconjugated 165kD-Vi (green circles) or comparable 42.7kD-fVi-CRM$_{197}$/165kD-Vi-CRM$_{197}$ conjugate as in first two immunizations (red circles). Representative dotplots of Vi^{+}B220^{+} B-cells among AnnexinV^{+} (*A*) or Annexin V^{neg} (*B*) in spleen 12h after booster at day 35 in mice immunized with two doses of 42.7kDa-fVi-CRM$_{197}$ or 165kDa-Vi-CRM$_{197}$. Vi^{+}B220^{+} B-cells *(C)* among lymphocytes in spleen 5 days after booster at day 35 in mice immunized with two doses of either 42.7kDa-fVi-CRM$_{197}$ (*light grey bars*) or 165kDa-Vi-CRM$_{197}$ (*dark grey bars*). Results expressed as (mean±SD) in spleen of 6-8 mice per group from one experiment; each dot corresponds to one mouse. Statistical difference was calculated using Mann-Whitney *U* test where comparable boosters were compared in mice immunized with two doses of 42.7kDa-fVi-CRM$_{197}$ or 165kDa-Vi-CRM$_{197}$. Representative dotplots of Vi^{+}B220^{+}B cells among IgM$^{+/neg}$ IgD^{neg} lymphocytes in BM 12h after each booster *(D)*.

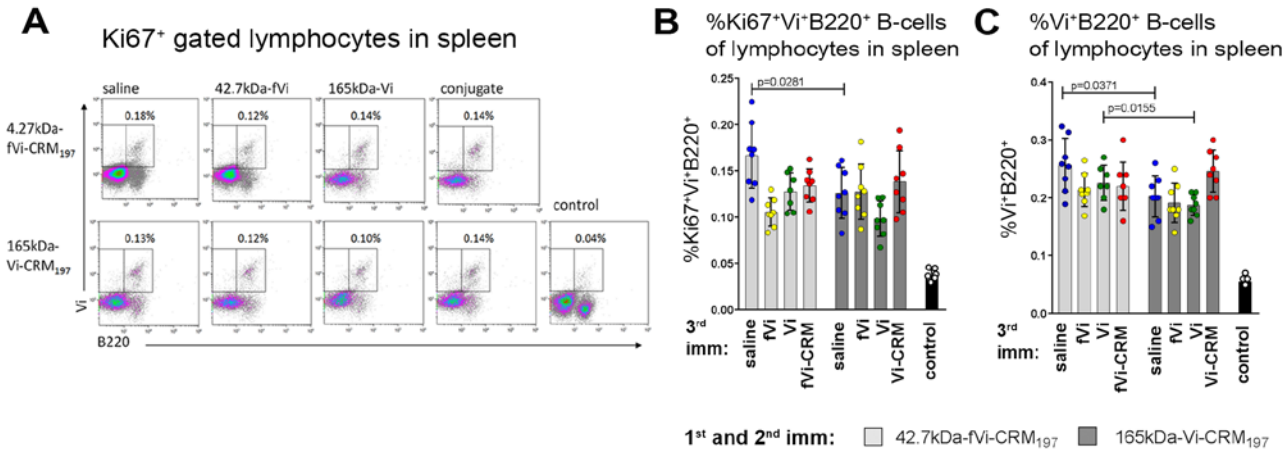

Fig. S6. The 42.7kDa-fVi-CRM$_{197}$ and 165kDa-Vi-CRM$_{197}$ conjugate induced comparable proliferation after 5 days but 42.7kDa-fVi-CRM$_{197}$ conjugate induction was more prolonged

The mice were immunized with two doses of 42.7kDa-fVi-CRM$_{197}$ or 165kDa-Vi-CRM$_{197}$ with 3 weeks interval and 2 weeks after the second dose the mice received different boosters, either saline (blue circles), unconjugated 42.7kD-fVi (yellow circles), unconjugated 165kD-Vi (green circles) or comparable 42.7kD-fVi-CRM$_{197}$/165kD-Vi-CRM$_{197}$ conjugate as in first two immunizations (red circles). Representative dotplots of Vi$^+$B220$^+$ B-cells among Ki67$^+$ lymphocytes in spleen 5 days after booster at day 35 in mice immunized with two doses of 42.7kDa-fVi-CRM$_{197}$ or 165kDa-Vi-CRM$_{197}$ *(A)*. Frequency of Ki67$^+$Vi$^+$B220$^+$ *(B)* and Vi$^+$B220$^+$ B-cells (*C*) among lymphocytes in spleen 5 days after booster at day 35 in mice immunized with two doses of either 42.7kDa-fVi-CRM$_{197}$ (*light grey bars*) or 165kDa-Vi-CRM$_{197}$ (*dark grey bars*). Results expressed as (mean±SD) in spleen of 7-8 mice per group from one experiment; each dot corresponds to one mouse. Statistical difference was calculated using Mann-Whitney *U* test where comparable boosters were compared in mice immunized with two doses of 42.7kDa-fVi-CRM$_{197}$ or 165kDa-Vi-CRM$_{197}$.

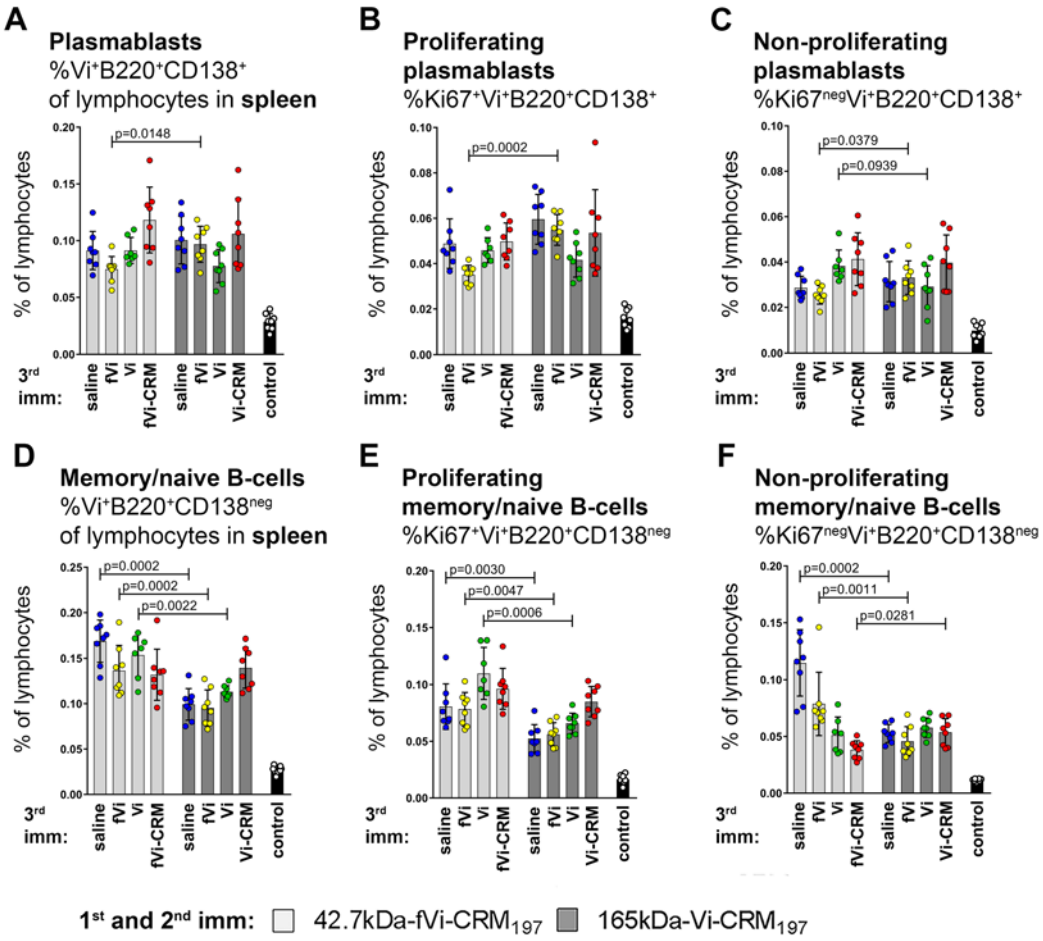

Fig. S7. Frequency of Vi-specific B-cell subpopulations in spleen 5 days after 3rd immunization. The mice were immunized with two doses of 42.7kDa-fVi-CRM$_{197}$ or 165kDa-Vi-CRM$_{197}$ with 3 weeks interval and 2 weeks after the second dose the mice received different boosters, either saline (blue circles), unconjugated 42.7kD-fVi (yellow circles), unconjugated 165kD-Vi (green circles) or comparable 42.7kD-fVi-CRM$_{197}$/165kD-Vi-CRM$_{197}$ conjugate as in first two immunizations (red circles). Frequency of Vi-specific B220$^+$ plasmablasts (Vi$^+$B220$^+$CD138$^+$)*(A)*, proliferating plasmablasts (Ki67$^+$Vi$^+$B220$^+$CD138$^+$) *(B)*, non-proliferating plasmablasts (Ki67^{neg}Vi$^+$B220$^+$CD138$^+$) *(C)*, memory/naïve (Vi$^+$B220$^+$CD138^{neg})*(D)*, proliferating memory/naïve (Ki67$^+$Vi$^+$B220$^+$CD138^{neg})*(E)* and non-proliferating memory/naïve (Ki67^{neg}Vi$^+$B220$^+$CD138^{neg})*(F)* among lymphocytes in spleen 5 days after booster at day 35 in mice immunized with two doses of either 42.7kDa-fVi-CRM$_{197}$ (*light grey bars*) or 165kDa-Vi-CRM$_{197}$ (*dark grey bars*). Results expressed as (mean±SD) in spleen of 7-8 mice per group from one experiment; each dot corresponds to one mouse. Statistical difference was calculated using Mann-Whitney *U* test where comparable boosters were compared in mice immunized with two doses of 42.7kDa-fVi-CRM$_{197}$ or 165kDa-Vi-CRM$_{197}$.

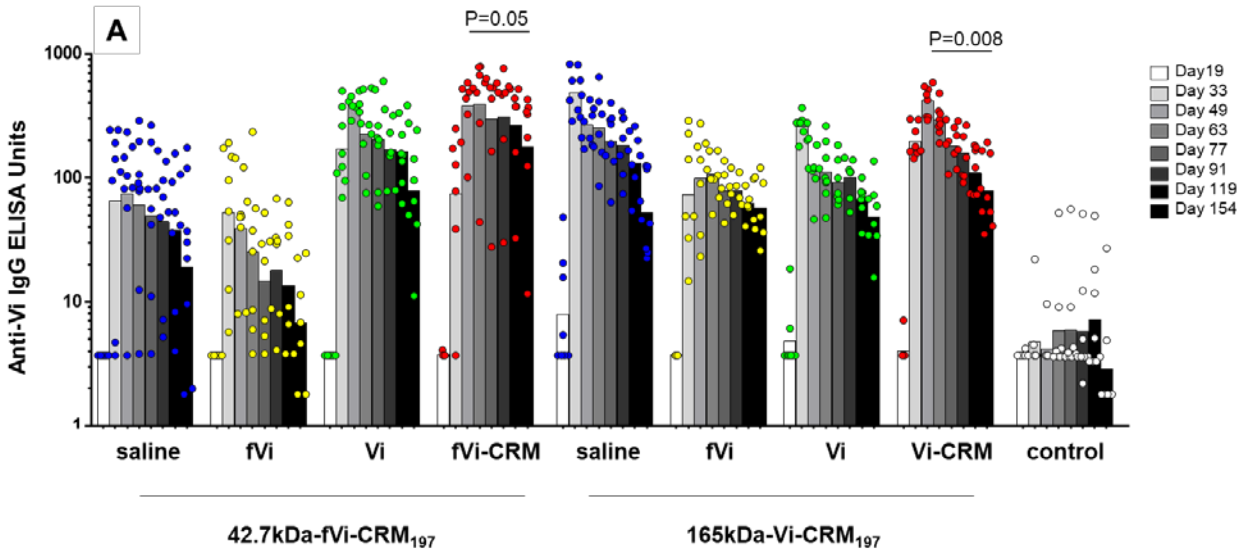

Fig. S8. 165kDa-Vi-CRM$_{197}$ vaccine induced less persistent antibodies compared to 42.7kDa-fVi-CRM$_{197}$.

The mice were immunized with two doses of 42.7kDa-fVi-CRM$_{197}$ or 165kDa-Vi-CRM$_{197}$ with 3 weeks interval and at day 35 received a booster with either saline (blue circles), unconjugated 42.7kDa-fVi (yellow circles), unconjugated 165kDa-Vi (green circles) or comparable conjugate as in first two immunizations (red circles). Vi-specific IgG response was measured in serum at day 19, 33, 49, 63, 77, 91, 119 and 154 after the 1st immunization with either 42.7kDa-fVi-CRM$_{197}$ or 165kDa-Vi-CRM$_{197}$. Individual animals are represented by the dots; column heights represent Geometric Mean Units. Statistical difference of antibody levels from day 49 to 154 was calculated using Wilcoxon matched-pairs signed rank test.