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ORIGINAL ARTICLE

Generation of oligomers of subunit vaccine candidate glycoprotein D of Herpes Simplex Virus-2 expressed in fusion with IgM Fc domain(s) in *Escherichia coli*: A strategy to enhance the immunogenicity of the antigen

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Abstract

Glycoprotein D (gD) of Herpes Simplex Virus-2 is used as an antigen in various anti-herpes subunit vaccines owing to its involvement in binding the host cell receptors for host infectivity. However, most of these monomeric protein based candidates have shown low immunogenicity in animal models. To enhance the immunogenicity of gD, a fresh approach of fusing its ectodomain with the Fc domain(s) of IgM has been adopted to oligomerize the viral antigen and to exploite the immune-modulating potential of IgM Fc. Six vaccine constructs, generated by fusing three gD-ectodomain-length-variants with the Ig µ-chain domain 4 (µCH4) and µCH3-CH4 fragment, were cloned in *Escherichia coli* using pET28b(+) vector. The vaccine proteins were expressed in the form of inclusion bodies (IBs) and were in vitro refolded into protein oligomers of high stoichiometries of ~ 15–24, with 70–80% refolding yields. The conformations of gD and Fc components of the refolded oligomers were analyzed by ELISA and CD spectroscopy and were found to be native-like. The sizes and profiles of the size-distribution of oligomers were determined by dynamic light scattering (DLS). The candidate C2 (gD-µCH3-CH4), showing the most compact oligomer size and uniform distribution of its particles was chosen as the suitable candidate for mice immunization studies to assess the immunogenicity of the antigen gD. The C2 oligomer stimulated a strong anti-gD humoral response with an antibody titer of 102,400 and a strong, biased Th1 immune response in C57BL/6 mice, indicating its potential as a strong immunogen which may serve as an effective vaccine candidate.

Keywords Glycoprotein D · Herpes simplex virus-2 · Fusion protein · Vaccine · In vitro refolding

Introduction

Herpes viruses are a leading cause of human viral diseases, second to influenza and cold viruses (Zhu and Zhu 2014). In the family *Herpesviridae*, at least eight virus types are known to infect humans. Among these, Herpes simplex virus-1 and 2 (HSV-1 and 2) also known as human herpesvirus-1 and 2, are the most prevalent worldwide. HSV-2 causes genital and neonatal herpes and is always transmitted sexually (Looker et al. 2015; Sauerbrei 2016). Primary infection of HSV-2 mostly progresses into a lifelong latent infection, often marked by one or more active outbreaks of the disease (White et al. 2012). Furthermore, HSV-2 infected individuals are generally at a higher risk of acquiring and transmitting HIV-1 infection (Wasserheit 1992; Wald and Link 2002). Till this date there is no vaccine or cure available for

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genital herpes. Among all types of vaccine formats, subunit vaccines remain the most tested prophylactic agents against herpes since the 80 s (Roth et al. 2013). Glycoprotein D (gD), an envelope glycoprotein, is required to bind to any one of its three known receptors viz. nectin-1, Herpesvirus entry mediator (HVEM), and heparan sulfate. Once the gD is bound to cellular entry receptor, it recruits other glycoproteins (gB, gH, and gL) and the concerted activity of these glycoproteins then leads to the fusion of virus with the host cell in an endocytic vesicle (Campadelli-Fiume et al. 2000; Spear et al. 2000). Therefore, gD has been used as the key immunogen in majority of the subunit vaccines, either as a single molecule or as one of the component of the combination formulations of other glycoproteins involved in virus entry, e.g. gB, gC or gG (Olson et al. 2009; Awasthi et al. 2011; Khodai et al. 2011; Skoberne et al. 2013). The subunit candidates although have been reported to elicit neutralizing immune-responses however they generally show low immunogenicity, often resulting in partial protection against the virus. Therefore different strategies had been adopted in the past to enhance the effectiveness of these candidates. Ye et al (2011) produced a chimeric candidate, by fusing the ectodomain of gD with Fc of IgG, aimed to stimulate the mucosal and systemic immunity and prolonging the systemic half-life of the candidate antigen for improving its immunogenicity. The fusion candidate was shown to generate protective antibodies and inhibit intravaginal infection of a virulent HSV-2 strain in mice (Ye et al. 2011). Since the envelope glycoprotein D has been reported to exists as a dimer and sometimes as an oligomer on the viral surface (Handler et al. 1996; Ye et al. 2011), therefore, a recombinant oligomeric gD, mimicking its native organization of the viral surface may elicit the desired protective immune response against HSV-2. Based on this hypothesis, expression of gD as a tetramer has also been attempted earlier by fusing it with a tetramerizing domain of transcription factor p53, wherein the multimeric candidate performed better in terms of eliciting immune response in animal models in comparison to the monomeric candidates (Perez-Hernandez et al. 2015).

In the current study, we report the fusion of ectodomain of gD with the Fc domain(s) of IgM to achieve oligomerization of the antigen with higher stoichiometry and immune modulation via IgM Fc receptor (FcμR)-Fc interactions between. In mouse, the IgM Fc receptor (FcμR) is predominantly expressed in B-cells (Shima et al. 2010; Ouchida et al. 2012; Liu et al. 2018); also in monocytes, macrophages, granulocytes and dendritic cells (Lang et al. 2013; Brenner et al. 2014) and has been shown to interact with and influence B-cell receptor signaling (Wang et al. 2016). In humans, FcμR is expressed by B, T, and NK cells and has been shown to modulate their immune responses (Kubagawa et al. 2009; Shima et al. 2010; Honjo et al. 2015). Furthermore, FcμR undergoes internalization after binding to IgM and hence a Fc-fusion biosimilar, a fusion vaccine candidate in the present case, may be taken up by the immune cells effectively, increasing its presentation and half-life inside the host (Vire et al. 2011).

Therefore, to generate chimeric vaccine candidates the ectodomain of gD was fused with constant heavy chain domain-4 (μCH4) and μCH3-CH4 Fc fragment of IgM to facilitate their organization as IgM-like oligomers (Choi et al. 2001; Müller et al. 2013). In total 6 candidates were generated by fusing 3 length-variants of gD with μCH4 and μCH3-CH4 fragments of IgM. Such a fusion of the gD ectodomain with immunoglobulin μ chain domains has been attempted for the first time in the current study. The fact that the glycosylation sites present on the native gD (N94, 121 and 262) do not participate in host receptor binding (Lu et al. 2014) or neutralizing antibody binding (Lee et al. 2013) and that the mouse Fc fragment μCH3-CH4 carries only three glycosylation sites (Anderson et al. 1985), makes the fusion proteins suitable to be expressed in a bacterial host which lacks post translational modification system. All vaccine proteins were expressed in *Escherichia coli* in the form of inclusion bodies and in vitro refolded to their oligomeric forms with 70–80% refolding yields. The candidate oligomers were further characterized using ELISA, CD spectroscopy and dynamic light scattering (DLS). Finally the candidate viz. C2 (gD-μCH3-CH4) was used to immunize the C57BL/6 mice to assess the immunogenicity of gD.

Materials and methods

The protein sequence of gD and IgM

The protein sequences of glycoprotein D were retrieved from the NCBI protein database using keywords- "*Herpes simplex virus 2 envelope glycoprotein D*". A total of 58 sequences were retrieved and the most commonly found sequence of gD was used (GenBank, AEA34987.1) for vaccine development [Supplementary information (SI) Table S1].

The murine immunoglobulin μ-chain DNA/ protein sequences were also retrieved from NCBI protein databank (GenBank accession no. AAB59650.1) (SI Table S1). Subsequently, the gD and IgM protein sequences were reverse translated into gene sequences online (https://www.bioinformatics.org/sms2/rev_trans.html) having *E. coli* codon bias and were thereafter chemically synthesized (Sigma Aldrich) as three separate DNA fragments viz. glycoprotein D (residues 1–315), μCH3 domain [residues 207–315], and μCH4 domain (330–454) containing extra 18 residues long linker peptide at its N-terminus [μCH2-CH3 interdomain linker peptide (residues 207–224)]. Later these three DNA fragments were joined in various permutation and combinations to generate six vaccine candidates (Fig. 1).

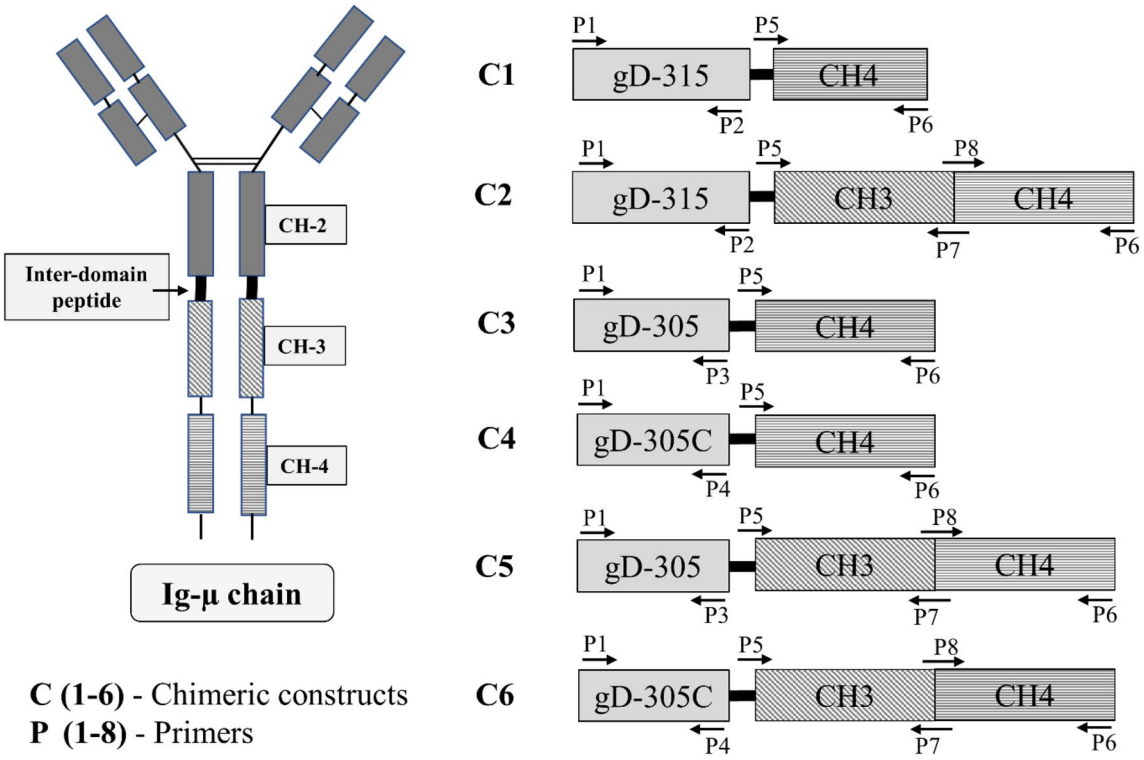

Fig. 1 Schematic representation of the design of the vaccine candidates with respect to their constituent domains. Primers (P1-P8) used to amplify individual gene fragments for cloning are depicted in the diagram with the help of arrows

Earlier studies have shown the flexible nature of N and C-terminus of gD (Carfí et al. 2001; Krummenacher et al. 2005; Di Giovine et al. 2011) and that the longer ectodomains (306 or 316 residues long) could not be crystallized (Carfí et al. 2001), until gD 306 was stabilized by the introduction of a disulfide bond at its C- terminus, which also favored its dimerization. Therefore, to screen the most suitable length of the antigen molecules, two different lengths of gD ectodomain, 305 and 315 residues, and a variant of 305 residues long fragment (with a cysteine residue as the terminal residue: gD305C) were selected as antigen molecules.

Assembly PCR and amplification of vaccine genes

The DNA fragments of glycoprotein D, μCH3, and μCH4 were amplified using restriction site introducing forward and reverse primers as listed in Table 1, following respective PCR program as provided in Table 2, in a total 50 μl reaction volume. To obtain joined μCH3-CH4 gene fragment, the

Table 1 The list of restriction-site-introducing primers used for PCR amplification and fusion of vaccine gene fragments

Primer code	Primer name	Sequence (5′-3′)
P1	gD_*Nde*I_For	ATGTTGATCATATGAAATATGCGCTGGCGGATC
P2	gD_315_*Not*I_Rev	ACATACATGCGGCCGCTGCCCGGGTTGCT
P3	gD_305_*Not*I_Rev	TAATACATGCGGCCGCTATGCGGCGCCACATCCT
P4	gD_305C_*Not*I_Rev	TAATACATGCGGCCGCTGCAATGCGGCGCCACATCCTG
P5	μCH3-CH4_*Not*I_For μCH4_*Not*I_For	GTATTGATGCGGCCGCTTTCTGAAAAACGTGAG
P6	μCH3-CH4_Stop_*Xho*I_Rev μCH4_Stop_*Xho*I_Rev	GAATCATACTCGAGTCAATAGCAGGTGCC
For assembly/joining of μCH3 and μCH4 gene		
P7	μCH3_Rev	CGGATGTTTATGCACTTCGTTCGGTTTGCTAATAAA
P8	μCH4_P_For	AAACCGAACGAAGTGCATAAACATCCGGCGGTGTATCTG

Table 2 The PCR programs for amplification of µCH3, µCH4, µCH3-CH4, gD-315, gD-305, gD-305C DNA fragements and assembly of µCH3 and µCH4 DNA fragments

Amplicon	Initial denaturation	30 cycles			Final extension	Hold
		Denaturation	Annealing	Extension		
µCH3	94 °C, 2 min	94 °C, 45 s	54 °C, 30 s	72 °C, 1 min	72 °C, 7 min	4 °C
µCH4			56 °C, 30 s			
µCH3-CH4			55 °C, 30 s			
gD_315		94 °C, 1 min	60 °C, 30 s			
gD_305			65 °C, 30 s			
gD_305C						
µCH3 and µCH4 assembly reaction	Initial denaturation	10 cycles			Final extension	Hold
		Denaturation	Annealing + Extension			
	94 °C, 2 min	94 °C, 30 s	60 °C, 10 min		72 °C, 7 min	4 °C

µCH3 and µCH4 DNA were amplified using primer sets P5/P7, and P6/P8, respectively. The equimolar concentrations of the µCH3 and µCH4 DNA (0.5 nM) were used in PCR reaction and subjected to assembly PCR for 10 cycles following a program as given in Table 2. The assembled template DNA was then amplified by PCR (SureCycler™ 8800; Agilent technologies) using the progamme as provided in Table 2, in a total 50 µl reaction volume. The PCR reaction mixture consisted of 10 ng template DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM forward and reverse primers and 1 U of *Taq* DNA polymerase (HiMedia).

Cloning of chimeric vaccine constructs

The glycoprotein D DNA fragments (gD-315, gD-305, and gD-305C) were cloned in pET28b (+) plasmid (Novagen) between *Nde*I and *Not*I restriction sites. The chimeric constructs were generated by subsequent cloning of µCH4/µCH3-CH4 gene fragments downstream of the respective gD gene between *Not*I and *Xho*I restriction sites. Briefly, the gD DNA fragments of different lengths were amplified by PCR using specific primers, as described earlier, and purified using a PCR clean-up kit (Macherey–Nagel). The purified samples of pET28b (+) and gD DNA were double digested using respective restriction enzymes (New England Biolabs) as per the manufacturer's protocol. The restricted DNA samples were gel extracted using a gel extraction kit (Macherey–Nagel). The vector (~ 50 ng) and insert DNA (~ 25 ng) fragments were ligated at a 1:3 molar ratio using T4 DNA ligase (New England Biolabs) as per manufacturer's instructions. Thereafter, the ligation mixture was heat-inactivated (65 °C for 20 min) and used to transform competent *E. coli* DH5α cells by heat shock method (Froger and Hall 2007). The transformed cells were spread over LB agar plates (supplemented with 50 µg/ml kanamycin) and incubated overnight at 37 °C. The colonies of the transformed cells were obtained the next day in case of all the vaccine candidates. A few colonies were randomly picked from each plate and screened for identification of recombinant clones by colony PCR and restriction digestion analysis of the recombinant plasmids, isolated from the selected clones those those produced positive vaccine gene amplification in the colony PCR. The presence of heterologous genes within recombinant plasmids was finally confirmed by DNA sequencing (Sanger sequencing) using T7 forward primer [TAATAC GACTCACTATAGGG] and T7 reverse primer [GCTAGT TATTGCTCAGCGG]. The DNA fragments encoding IgM domain(s) encoding were then cloned downstream gD as per each vaccine design. After cloning all the components of vaccine candidates, each vaccine chimeric clone was re-sequenced, confirming its correct sequence and reading frame.

Protein expression

E. coli BL21 (DE3) cells were transformed with the recombinant plasmids carrying vaccine constructs, (e.g., pET28-gD-315- µCH4 and others) and plated on kanamycin supplemented LB-agar plates. The next day, 5 ml of LB media (supplemented with kanamycin (50 µg/ml) was inoculated with a single colony and incubated at 37 °C in an orbital shaker overnight, with continuous shaking at 150 rpm. 400 ml TB media (supplemented with 50 µg/ml kanamycin) was inoculated with 4 ml (1%) of the overnight grown culture and allowed to grow till OD_{600} of ~ 0.8 at 37 °C. The culture were induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and after that allowed to grow in an orbital shaker at 37 °C for 6 h with continuous shaking at 200 rpm. Cells were harvested and resuspended in ice-cold sonication buffer (50 mM Tris–HCl, 5 mM EDTA, 1 mM PMSF, pH 8.0). Cultured cells were lysed by sonication (30-s burst phase/30-s rest phase/ 130 W) through 8 cycles, using Ultrasonic Processor (Cole-Parmer). The heterologous proteins in the

lysate were analyzed by SDS-PAGE and western blotting using anti-6xHis-tag antibody. Briefly, the total cell lysates of induced cultures were subjected to SDS-PAGE. The SDS-PAGE resolved protein bands were transferred onto a nitrocellulose membrane (NC) for western blotting. The NC membrane was blocked with 5% BSA in PBS and incubated for 1 h at room temperature followed by a single wash with PBS containing 0.05% Tween-20 (PBST). 10 ml of anti-6xHis-tag primary antibody (#SAB2702220, Sigma-Aldrich), at 1:20,000 dilution in PBST, was added to the blocked NC membrane and incubated for 1 h at room temperature with slow stirring. The membrane was washed thrice with PBST and then incubated with 10 ml of secondary antibody (#A0168, Sigma-Aldrich), at 1:180,000 dilution in PBST, for another 1 h. Post PBST washing thrice, 10 ml of ready to use TMB (3,3′,5,5′-Tetramethylbenzidine) substrate solution (Sigma-Aldrich) was added to the NC membrane and it was incubated in a dark room for 20 min for the appearance of the desired bands.

Isolation of inclusion bodies (IBs) and protein purification

The induced cultures of recombinant *E. coli* BL-21 (DE3) cells were harvested and sonicated as described above. The resulting cell lysates carrying heterologous proteins were centrifuged at 12,000 g for 30 min at 4 °C to obtain a compact pellet containing IBs. The pellets were washed twice, first with washing buffer (1% sodium deoxycholate, 2% Triton X-100, 50 mM Tris–HCl, 5 mM EDTA, pH 8.0) and then Milli Q water, wherein the IBs were resuspended by performing sonication for 2 cycles and subsequent slow mixing for 30 min on a tube rotator. After each resuspension-washing cycle, the IBs were harvested by centrifugation at 12,000 g for 30 min at 4 °C.

The washed IBs pellets of vaccine candidates were solubilized in buffer A (8 M Urea, 50 mM Tris–HCl, pH 8.5) for purification. The chimeric vaccine constructs were purified by anion-exchange chromatography using DEAE sepharose resin (GE Healthcare). The IBs solubilized in buffer A were centrifuged and the supernatants were loaded on to the columns, pre-equilibrated with buffer A. Post 5 column volume wash with buffer B (8 M Urea, 50 mM Tris–HCl, 5 mM NaCl, pH 7.0), column bound protein was eluted using buffer C (8 M Urea, 50 mM Tris–HCl, 50 mM NaCl, pH 7.0). The eluted samples were analyzed by 12% SDS-PAGE and the purified elution fractions were mixed together and the protein concentration of the resulting pooled samples was estimated by Bradford assay. The percent purity of all the constructs was estimated by densitometry using software Image J.

In vitro refolding

In vitro refolding of purified and denatured vaccine proteins was performed according to the cited method with slight modification (Tapryal et al. 2010). The purified and pooled protein samples were diluted to a concentration of 0.5 mg/ml. β-mercaptoethanol was added to the protein samples at a concentration of 10 mM and incubated with slow stirring/mixing at room temperature for 1 h. The reduced protein samples (10 ml, 0.5 mg/ml) were transferred to a dialysis bag (10 kDa cutoff) and subjected to dialysis against 250 ml of buffer G (8 M urea, 50 mM NaCl, 1 mM EDTA, and 50 mM Tris, pH 8.0) at 4ºC with continuous slow stirring of the exchange buffer, to remove the reducing agent. The concentration of urea was then reduced in successive dialysis steps using the dialysis-exchange-buffers containing gradually decreasing concentration of urea, 8 M → 6 M → 4 M and then → 2 M. The dialysis bag was then transferred to buffer H (1 M urea, 400 mM L-arginine, 50 mM NaCl, 1 mM EDTA, 375 µM oxidized glutathione and 50 mM Tris, pH 8.0) containing oxidizing agent and thereafter urea was removed completely in next four successive steps (1 M → 0.5 M → 0.25 M → 0 M). In a similar fashion, L-arginine and oxidized glutathione were also removed in the subsequent four steps. Post complete removal of Urea, L-arginine and oxidized glutathione the final dialysis step was performed against buffer I (50 mM NaCl, 50 mM Tris, pH 8). The duration of each dialysis step was about 6–8 h performed at 4 °C with continuous slow stirring of the exchange buffer. The in vitro refolding yield was calculated using the given formula

$$\text{Refolding yield} = \frac{\text{Total recovered refolded protein (mg)}}{\text{Initial protein used for refolding (mg)}} * (100)$$

ELISA

Freshly refolded vaccine proteins were analyzed using ELISA. The candidate proteins were diluted in the carbonate-bicarbonate buffer (50 mM, pH 9.6)/ PBS (100 mM, pH 7.4) to a final concentration of 10 µg/ml. 100 µl of the above mentioned solution was added to the desired number of wells of microtiter plate to coat 1 µg of vaccine protein. Vaccine proteins were coated in triplicate and plate was incubated at 4 °C overnight. Next day the coated wells were blocked with 5% BSA solution (prepared in PBS) for 2 h at 37 °C in a humid chamber. Post single wash of the well with PBST (PBS containing 0.05% Tween-20), 100 µl of diluted (1:1500) anti-gD primary antibody E317 (#MAB12121, The Native Antigen Company) solution, prepared in PBST, was added to the wells and the plate was incubated for 2 h at

37 °C in a humid chamber. The wells were then washed thrice with PBST. Thereafter, the microwells were incubated with 100 µl of diluted (1:10,000) goat anti-mouse secondary-antibody (#A0168, Sigma-Aldrich) solution, prepared in PBST, for 2 h at 37 °C in a humid chamber. The wells were then washed thrice with PBST and incubated with 100 µl of TMB substrate ready-to-use solution (1x) in a dark room at room temperature for 20 min. Thereafter, 25 µl of 2 M H_2SO_4 stop solution was added to each well and the absorbance was recorded at 450 nm wavelength using microtiter plate reader (Thermo).

To probe vaccine proteins for binding goat-anti-mouse IgM Fc antibody, the antigen proteins (vaccine oligomers) were coated overnight and blocked with 5% BSA as mentioned in above paragraph. After a single wash, the microwells were incubated with 100 µl of diluted (1:5000) anti-mouse IgM(Fc) HRP linked antibody (#1021–05, Southern Biotech) solution prepared in PBST, for 2 h at 37 °C in a humid chamber. Thereafter, the wells were washed thrice and incubated with 100 µl of TMB substrate solution followed by addition of H_2SO_4 and absorbance values were recorded at 450 nm wavelength as described above.

Size exclusion chromatography (SEC)

The oligomeric vaccine proteins obtained post in vitro refolding were segregated from their respective monomeric forms by size exclusion chromatography. The SEC was performed on a 16/600 Superdex 200 pg prepacked column of 120 ml volume, using AKTA™ pure system (GE Healthcare) at a flow rate of 1 ml/min in buffer system (50 mM Tris, 150 mM NaCl, pH 8.0). The elutes were monitored by inbuilt UV 280 nm absorbance apparatus (pathlength: 0.2 cm). A 120 ml column was calibrated with high molecular weight marker kit (GE Healthcare) to plot the standard curve and determine the molecular weights of the vaccine oligomers corresponding to their elution peaks. The data of SEC were analyzed by software UNICORN 7.0 and the graphs were plotted using software Origin 8.5.

Endotoxin quantification LAL assay

The vaccine proteins were expressed in bacterial host and hence the presence of the bacterial endotoxins in the vaccine preparations were assessed using LAL (Limulus amebocyte lysate) assay. The assay was performed using the endotoxin test kit (Thermo Scientific) by following the protocol provided by the manufacturer. Briefly, 50 µl volume each of the test and diluted standard endotoxin samples, in the range of 0.1–1.0 endotoxin unit (EU)/ml, was added into the wells of a 96 well microplate (in duplicates), pre-equilibrated at 37 °C. 50 µl of LAL reagent was then added into each well and incubated for exactly 10 min at 37 °C. Further, 100 µl of the chromogenic substrate solution was added to each well and incubated for exactly 6 min at 37 °C. After 6 min, 100 µl of stop reagent (25% acetic acid) was added into each well and the absorbance was recorded at 405 nm using microtiter plate reader (Multiskan GO, Thermo Scientific). A consistent pipetting speed and order of reagent addition in the wells was maintained, especially during the last two steps, to achieve uniform incubation timings in each well. A standard graph of absorbance units *versus* endotoxin units of the standard endotoxins was plotted and subsequently used to determine the concentrations in the test samples, corresponding to their absorbance values/units. One EU is equivalent to 100 pg of bacterial endotoxin/ml of solution. For a recombinant subunit vaccine, the endotoxin level permitted for immunizing animals is recommended to be < 20 EU/ml (Brito and Singh 2011).

Circular dichroism (CD) spectroscopy

To collect CD data of the vaccine proteins, the buffer of the protein samples were changed (from 50 mM Tris, 150 mM NaCl, pH 8.0 buffer to 50 mM sodium phosphate buffer, pH 7.4) using Amicon centrifugal filters (Merck Millipore) and during this step the samples were also concentrated for their protein content. The far-UV CD spectra (200–260 nm) of vaccine candidates (at protein concentration of 0.3–0.5 mg/ml) were recorded using the Chirascan CD spectrometer. The final average spectra were measured by subtracting the baseline spectra of the buffer. The molar ellipticity of the samples was plotted against wavelength to obtain the secondary structure-specific plots/graphs. The molar ellipticity was calculated using the following formula

$$\text{Molar ellipticity } (\theta) = \frac{(m^\circ * M)}{(10 * L * C)}$$

where, θ (theta) represents molar ellipticity in deg.cm^2. dmol^{-1}, m° is recorded ellipticity in millidegrees, M specifies mean residual weight (g/mol), L is pathlength in cm, and C is concentration in g/L. The secondary-structure elements of the vaccine oligomers were predicted by analyzing their CD-spectra data using CAPITO software online (https://capito.nmr.leibniz-fli.de//).

Dynamic light scattering (DLS)

The diameter of the protein oligomers was analyzed on a Malvern Zetasizer Nano™ instrument using disposable cuvettes at room temperature. The obtained particle size diameter was used to calculate the hydrodynamic radius of the protein oligomer using formula Rh = d/2; where, Rh- hydrodynamic radius, and d- average diameter of protein oligomers. The polydispersity index (PDI), which is a

measure of broadness of molecular weight distribution of an analyte in solution, was also considered while choosing the best candidate among all. A lower PDI value is indicative of a narrower range of the size distribution of the analyte, refolded proteins in the present case. The PDI value of vaccine constructs were calculated by the Malvern Zetasizer Nano™ instrument software.

Immunization of C57BL/6 mice

Clinically healthy, 3–4 weeks old female C57BL/6 mice were procured from CSIR-Indian Institute of Integrative Medicine, Jammu, India and housed at the animal-house-facility of the University of Rajasthan, for further studies. Approval of the Institutional Animal Ethical Committee (IAEC) of the host university was obtained prior to performing the immunization experiments and all the guidelines and regulations of the IAEC were complied with during the study. The detail of the approval for carrying out the mice immunization studies is provided in the "Compliance with ethical standards" section. Mice were segregated in different groups and allowed to acclimatize for 2 weeks at the facility. Mice were kept in the groups of 6 in polypropylene cages (size $43 \times 27 \times 15$ cm) with 12:12 h light and dark cycles. Animals were fed with autoclaved mice pellet diet (M/s Ashirwad Industries Limited, Chandigarh, India) and water ad libitum. The temperature of animal house was maintained at 25 ± 2 °C with relative humidity 40–70%. At an age of 5–6 weeks (weighing 22–25 g), mice were subjected to the immunization with vaccine candidate/adjuvant emulsified formulations. During the first immunization, each mouse of the test groups was immunized with 20 µg vaccine antigen using 0.45×13 mm needle (DISPO VAN®, India). A mixture of 1:1 antigen to adjuvant ratio, in 100 µl volume, was emulsified by repeatedly passing it through 22-gauze needle till the prepared emulsion forms a non-dispersive drop of emulsion on water. The first dose of the vaccine in Freund's complete adjuvant (FCA) (Sigma-Aldrich, #F5881) was given subcutaneously (day 1). The subsequent two booster doses (on day 14 and 28) were administered intraperitoneally in Freund's incomplete adjuvant (FIA) (Sigma-Aldrich, #F5506) at an interval of two weeks from the previous dose. Thereafter, the third booster was given intraperitoneally (on day 42) in PBS and on fourth-day (day 45) post-immunization mice were sacrificed. The control group mice were immunized with the emulsion of sterile PBS and respective adjuvants and third booster was administered with only sterile PBS. The physical health of the mice was monitored closely. The blood samples of mice were collected by tail bleeding, 4 h before every immunization viz. on day 1, 14, 28, 42 and 45. The serum from the collected blood samples was isolated and was stored at -20ºC for further analyzed.

ELISA for antibody titer

For coating all the vaccine proteins, a total of 100 µl of each of the antigen (10 µg/ml) prepared in carbonate-bicarbonate (50 mM, pH 9.6) antigen coating buffer was coated in microwell plate in duplicates and incubated at 4ºC overnight. 100 µl of 5% BSA in PBS was added to each well and plate was incubated for 2 h at 37 °C in a humid chamber. After a single wash with PBST (containing 0.05% Tween-20), the wells were incubated with 100 µl of two-fold serially diluted (in PBST) serum samples of the test and control groups, for 2 h at 37 °C in a humid chamber. Thereafter the wells were washed thrice with PBST. The microwells were then incubated with 100 µl of diluted (1:10,000) goat anti-mouse-IgG secondary antibody (#A0168, Sigma-Aldrich) solution prepared in PBST, for 2 h at 37 °C in a humid chamber. Subsequently, wells were washed thrice with PBST and then the steps involving incubation with substrate, stopping of the reaction and reading of the absorbance values, were performed as described above. The reciprocal of the highest serum-dilution that produces absorbance units at least three times higher than the control is designated as the antibody titer.

To estimate the IgM antibody titer during the immunization schedule, the antigen (SEC purified gD-315/gD-315-µCH3-CH4) was coated overnight at 4 °C and blocked with 5% BSA as described earlier. After a single wash with PBST, the wells were incubated with 100 µl of two-fold serially diluted (in PBST) serum samples of the test and control groups at 37 °C in a humid chamber for 2 h. Post washing (3 times), the wells were incubated with 100 µl of 1:5000 diluted anti-mouse IgM (Fc) HRP antibody (#1021–05, Southern Biotech), prepared in PBST and incubated for 2 h at 37 °C in a humid chamber. Further, the wells were washed thrice with PBST and thereafter 100 µl of TMB substrate solution was added and incubated for 20 min. Subsequently, to stop the reaction 2 M H_2SO_4 solution was added and the absorbance values were recorded at 450 nm wavelength as described in above paragraph.

Isolation and culturing of splenocytes

The C57BL/6 mice were sacrificed on day 45 and their spleens were removed aseptically. The isolated spleens were washed in cold PBS and teased gently against a pair of sterile frosted glass slide to dislodge the cell mass in RPMI 1640 medium. The resulting splenocyte suspension was washed in PBS and cells were collected by centrifugation at 200 g for 5 min at 4 °C. The pellet obtained was resuspended in RBC lysis buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, and 0.1 mM EDTA) and incubated for 10 min on ice and cells were collected by centrifugation at 200 g for 5 min at 4 °C. Thereafter the cell pellet was washed

in RPMI 1640 medium and finally resuspended in 2 ml of same medium with 10% fetal calf serum. For counting the viable cells, the diluted cell suspension was mixed with 0.4% trypan blue in 1:1 ratio and the mixture was then loaded onto a hemocytometer wherein cells were counted under microscope. The final splenocyte count was adjusted to 1.0×10^6 cells/ml. The vaccine sample used in the culture studies had C2 and endotoxin stock concentrations of 0.7 mg/ml and 3.2 EU/ml, respectively. 1 ml of splenocyte suspension in RPMI 1640 medium supplemented with 10% serum and 20 µg/ml (0.08 EU/ml endotoxin) of test antigen C2 or positive control antigen [5 µg/ml Concanavalin A (Con A)] was added into wells of a 24 well cell-culture plate and incubated in 5% CO_2 incubator at 37 °C for 72 h. The cell supernatants were collected at 36 and 72 h by centrifugation at 5000 g at 4ºC for 10 min and stored at – 80 °C for cytokine analysis.

Cytokine assay

The culture supernatant of the splenocytes, isolated from control and test group mice were analyzed for determining the type of cytokines released by them under the stimulation of the vaccine antigen. Th1/Th2 cytokine assays were performed using Thermo Scientific ELISA kits (#888,350 TGF-β, #885,019 IL-1α, #887,137 IL-13, #887,324 TNF-α, #88,771,144 IL-2, IL-4, IL-10, IFN-γ). The assay procedures were performed at room temperature in duplicates as per the manufacturer's manual. Briefly, 100 µl of diluted capture antibody in coating buffer was added into microwells and incubated overnight at 4 °C. The coated capture antibody was aspirated out and wells were washed thrice with wash buffer. The wells were blocked using ELISA diluent provided in the kit and incubated for 1 h. The reagents were aspirated out and wells were washed once with wash buffer. Subsequently, 100 µl of twofold serially diluted standards and culture supernatant samples were added to respective wells and incubated for 2 h. The samples were aspirated out and wells were washed thrice again. Thereafter, 100 µl of detection antibody was added per well and incubated for 1 h. The detection antibody was aspirated out and the wells were washed thrice. Then, 100 µl of avidin-HRP was added per well and incubated for 30 min. The added avidin-HRP solution was aspirated and wells were washed 5 times. Finally, 100 µl of TMB solution was added per well and incubated for 15 min, followed by addition of 50 µl stop solution per well. The absorbance was recorded at 450 nm using the microplate reader. A standard graph was plotted with cytokine concentration presented on x-axis and absorbance on y-axis to determine the concentration of a particular cytokine in the sample.

Statistical analysis

The statistical significance in all the experiments was accepted at $P < 0.05$. ANOVA (Analysis of Variance) was used for statistical analysis. Tukey's multiple comparisons test was used to compare the control and test samples. The statistical analyses were performed using the software GraphPad Prism 6.0.

Results

Cloning and expression of vaccine proteins in *E. coli*

The vaccine candidates are designed as chimeric molecules consisting of an N-terminal viral glycoprotein D ectodomain and a C-terminal IgM Fc domain(s). The C-terminus Fc fragments (µCH4 or µCH3-CH4) are the oligomerizing units of the vaccine proteins facilitating their assembly into IgM-like pentamers or hexamers. Theoretically thus, a vaccine protein pentamer or hexamer would contain ten or twelve monomeric units respectively. To produce vaccine proteins in *E. coli*, six clones of vaccine chimeric genes (C1-C6) were generated by recombining sequentially the gD and IgM Fc DNA with pET28b (+) expression vector as described in materials and methods section (Fig. 1 and Table 3). The insertion of heterologous genes in the recombinant plasmids was confirmed by colony and standard PCRs, restriction digestion analysis and DNA sequencing. Vaccine clones showed expected DNA sequences wherein no mutation/reading-frame-shift was observed in the deduced sequences (SI, Table S2). The vaccine constructs (C1-C6) were expressed with a vector encoded 20 residues long peptide (MGSSHHHHHHSSGLVPRGSH) at their N-terminus carrying a 6xHis tag. The vaccine proteins were expressed in *E. coli* BL21 (DE3) cells under the induction of IPTG. The harvested cells were lysed by sonication and samples were analyzed by SDS-PAGE for expression of heterologous proteins. The induced bands of vaccine proteins were observed migrating alongside corresponding molecular weight marker bands on SDS-PAGE, indicating the expression of hetrologous proteins (Fig. 2a). The expressed vaccine proteins (C1-C6) accumulated inside *E. coli* cytoplasm in the form of insoluble inclusion bodies, as indicated by co-localization of the heterologous proteins-bands with that of the pellet fraction of the cell lysate samples on SDS-PAGE (Fig. 2b). Furthermore, cell lysate samples of the induced *E. coli* cultures expressing vaccine proteins were analyzed by western blotting using anti-6xHis-tag mAb. As shown in Fig. 2b and c, in addition to the appearance of induced vaccine protein bands, a few extra bands of lower molecular weights were also observed across all samples on SDS-PAGE and western blot, suggesting the latter to be the

Table 3 Description about the nomenclature and the design of the chimeric vaccine proteins

Vaccine construct code	Construct name	Cloning site in pET28(b) + vector	Construct description
C1	gD-315-μCH4	*Nde*I – *Not*I—*Xho*I	(1–315, glycoprotein D ectodomain) + [(μCH2-CH3 interdomain peptide linker) + (μCH4)]
C2	gD-315-μCH3-CH4	*Nde*I – *Not*I—*Xho*I	(1–315, glycoprotein D ectodomain) + [(μCH2-CH3 interdomain peptide linker) + (μCH3-CH4)
C3	gD-305-μCH4	*Nde*I – *Not*I—*Xho*I	(1–305, glycoprotein D ectodomain) + [(μCH2-CH3 interdomain peptide linker) + (μCH3-CH4)]
C4	gD-305C-μCH4	*Nde*I – *Not*I—*Xho*I	[(1–305, glycoprotein D ectodomain) + cysteine] + [(μCH2-CH3 interdomain peptide linker) + (μCH4)]
C5	gD-305-μCH3-CH4	*Nde*I – *Not*I—*Xho*I	(1–305, glycoprotein D ectodomain) + [(μCH2-CH3 interdomain peptide linker) + (μCH3-CH4)]
C6	gD-305C-μCH3-CH4	*Nde*I – *Not*I—*Xho*I	[(1–305, glycoprotein D ectodomain) + cysteine] + [(μCH2-CH3 interdomain peptide linker) + (μCH3-CH4)]

truncated products of the vaccine proteins. Upon analyzing the protein sequence of gD, it was found that its ectodomain contains six, Asp-Pro (D-P) peptide bonds which are 8–20 times more labile than D-X (Asp-other than proline residues) bond and 100 times more labile than peptide bond lacking aspartate residue (Tsung et al. 1989; Volkin et al. 1997). Moreover, the observed accumulation of the extra protein bands was found proportional to the size of the vaccine proteins, as higher amounts of truncated proteins were observed to have co-expressed with the large sized chimeric constructs (Fig. 2c), suggestive of incomplete translation of heterologous-proteins under IPTG induction. In order to reduce the co-expression of truncated protein-fragments, the C2 expressing bacterial cells were cultured at lower temperature of 30ºC, wherein a marginal improvement in the level of full-length heterologous-protein was observed, however the amount and the pattern of the truncated protein-fragments remained unaltered (SI, Fig. S1). On the other hand, the total wet biomass of the culture obtained at 30 °C, 6 h post induction, was marginally lower than what was obtained at 37 °C (data not shown). Additionally, the process of protein production at lower temperature was found to be time consuming as the cell cultures were required to acclimatize to the lower temperature prior to induction, which introduced additional step and a time lag of about 30 min in the process. Therefore, the recombinant cells expressing vaccine proteins were cultured at a temperature of 37ºC for large scale production.

Purification and in vitro refolding of vaccine proteins

The vaccine proteins were expressed as inclusion bodies inside *E. coli* cytoplasm. The IBs were solubilized using buffer containing 8 M urea and then purified by anion exchange chromatography as described in the methods section. Since the truncated vaccine proteins partially shared the physicochemical properties of their corresponding full-length counterparts, the two could not be separated completely. As a result, considering the truncated products as contaminants, the full-length vaccine proteins could only be purified with 70–75% purity (Fig. 2d). The purity of the protein samples was estimated using Image J software (Table 4). The partially purified heterologous proteins were then immediately subjected to in vitro refolding using a method that employs slow and differential removal of the chaotropic and oxidizing agents, as described in methods section. The refolded protein samples were analyzed by SDS-PAGE under reducing and non-reducing conditions wherein, in the latter case, all the vaccine proteins showed upward shift in their band positions and remained immobilized in the wells of the gel (Fig. 2d and e), suggesting their oligomerization into complexes of higher molecular weights. Furthermore, presence of non-associated, monomeric vaccine proteins in the refolded samples (Fig. 2e), indicated the oligomerization to be a stoichiometry driven process. All the candidate proteins were refolded with yields ranging between 75–85%. A stepwise account of expression, purification, in vitro refolding and secondary structure composition of all the six vaccine candidates has been summarized in Table 4.

Validation of native conformation of vaccine proteins by ELISA

To validate the proper three-dimensional conformation of the in vitro refolded vaccine proteins, their binding with anti-gD monoclonal antibody (mAb) E317 and anti-IgM Fc polyclonal antibodies was assessed by ELISA. All the oligomeric vaccine proteins showed binding to both the antibodies. The mean absorbance data obtained during ELISA was compared with the negative control (no antigen) by applying the one-way analysis of

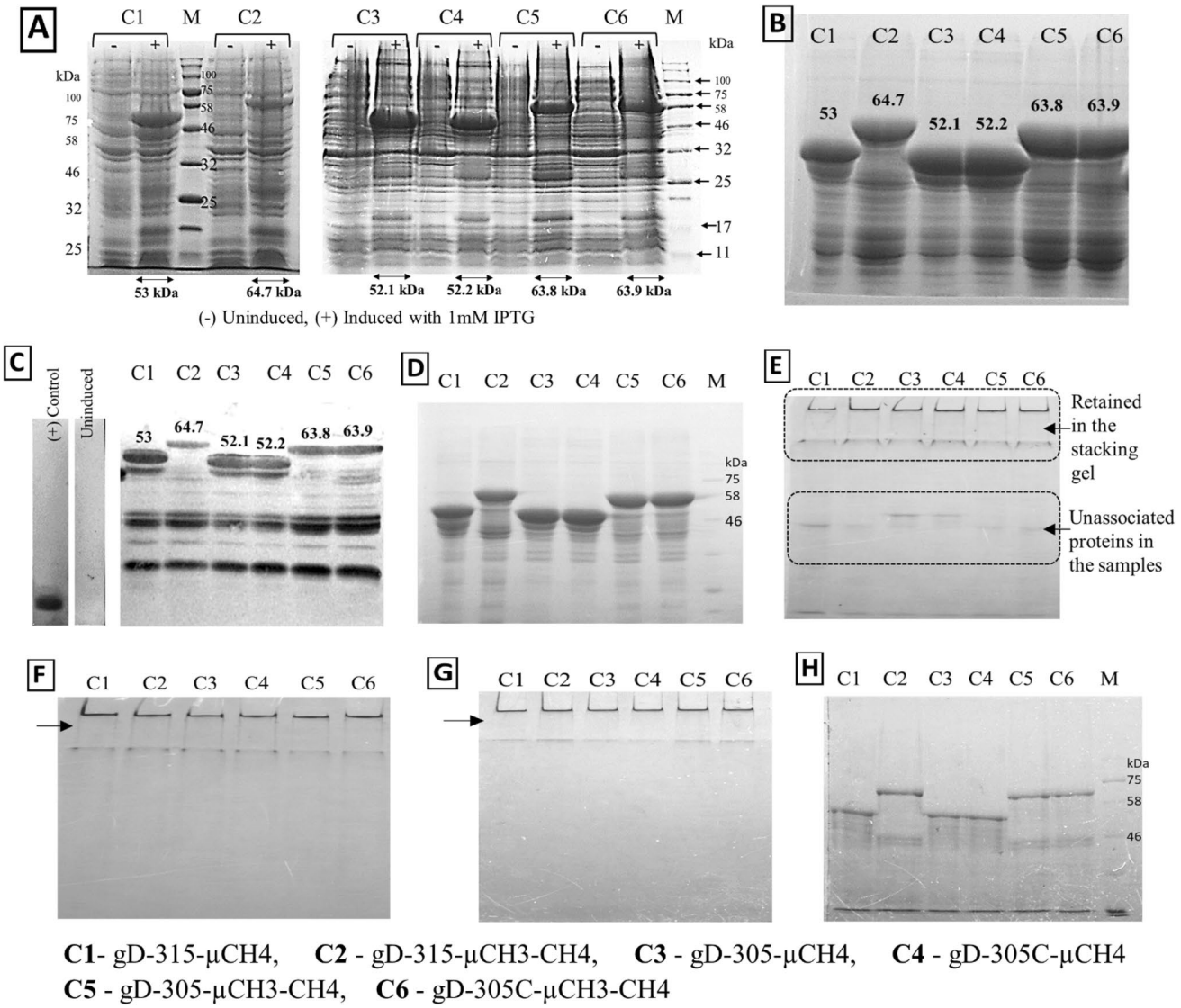

Fig. 2 PAGE and western blot analysis of vaccine proteins C1-C6. **a** SDS-PAGE analysis of the normalized whole-cell-lysate samples of the induced and un-induced *E. coli* BL-21 (DE3) recombinant cells, transformed with respective vaccine clones (C1-C6). The un-induced samples were collected just before induction, whereas the induced samples were collected 6 h post IPTG induction. **b** SDS-PAGE analysis of the isolated and washed vaccine inclusion bodies (IBs). The *E. coli* BL-21 (DE3) cells expressing vaccine proteins were sonicated and IBs were collected by centrifuging the cell lysate. The IBs were then treated/washed with sodium deoxycholate/Triton X-100 and the resultant samples were subjected to SDS-PAGE. **c** Western blot analysis of the whole-cell-lysate samples of the induced *E. coli* BL-21 (DE3) cells, expressing vaccine proteins. The samples were run on SDS-PAGE and the resolved protein bands on the gel were transferred on to nitrocellulose membrane, which was thereafter probed using anti-6xHis-tag antibody to detect the presence of heterologous-proteins in the lysate samples. **d** SDS-PAGE analysis of the purified vaccine proteins C1-C6. The washed vaccine IBs were re-suspended in the buffer carrying 8 M urea and subjected to anion exchange chromatography. The purified pooled-samples were subjected to SDS-PAGE. **e** SDS-PAGE analysis: The in vitro refolded samples of the vaccine proteins C1-C6, were mixed with loading buffer deficient in reducing agent β-mercaptoethanol and were subjected to SDS-PAGE. Under the non-reducing conditions, the oligomers of the vaccine proteins were retained in the stacking gel, whereas the bands of unassociated monomeric-vaccine-proteins were observed in the resolving gel. The samples of the in vitro refolded and SEC purified vaccine proteins, C1-C6, were mixed with loading buffer deficient in reducing agent β-mercaptoethanol and were subjected to **f** Native-PAGE, **g** SDS-PAGE, wherein all the vaccine oligomers were retained in the wells of the gel and no other protein bands were observed in the resolving gel, indicating high purity of the oligomeric vaccine proteins. **h** SDS-PAGE analysis: The samples of the in vitro refolded and SEC purified vaccine proteins, C1–C6, were subjected to SDS-PAGE under reducing conditions. The constituent subunits of the vaccine oligomers were resolved as distinct bands on the gel, indicating the participation of both the full length and the truncated vaccine proteins in the oligomer assembly

Table 4 Summarized information about the expression; purification; in vitro refolding; molecular weight, stoichiometry, secondary structure profile, hydrodynamic radius and polydispersity index of the vaccine proteins

Construct code	C1	C2	C3	C4	C5	C6
Construct name	gD-315-μCH4	gD-315-μCH3-CH4	gD-305-μCH4	gD-305C-μCH4	gD-305-μCH3-CH4	gD-305C-μCH3-CH4
Wet weight of biomass per 1000 ml of culture (grams)	3.425	3.095	3.335	3.365	3.255	3.24
Wet weight of washed inclusion bodies (IBs) per 1000 ml of culture (gm)	0.355	0.305	0.38	0.345	0.32	0.345
% purity of IBs	35	30	35	35	30	30
Purified protein (mg)	24	19	22	25	19	20
% purity of vaccine proteins	75	70	75	75	70	70
Refolded protein (mg)	20	16	18	21	16	17
% refolding yield	83.3	84.2	81.8	84	84.2	85
Molecular weight of oligomeric candidate protein estimated by SEC (kDa) (O)	984.66	984.66	1178.15	1188.3	1184.23	1220.35
Calculated molecular weight of monomeric candidate protein (kDa) (M)	53.08	64.75	52.18	52.28	63.85	63.95
Stoichiometry of oligomeric candidate protein [(estimated mol wt of vaccine proteins (O)/ calculated mol wt (M)]	18.55	15.21	22.58	22.73	18.55	19.08
Estimated content of secondary structure (%)						
α-helix	3.7	3.7	3.8	3.7	4.0	3.7
β-sheet	32.9	32.9	32.8	32.8	32.9	32.9
Hydrodynamic radius (nm)	93.55	38.225	58.75	47.48	49.075	102.75
Polydispersity index (PdI)	0.384	0.195	0.371	0.356	0.261	0.305

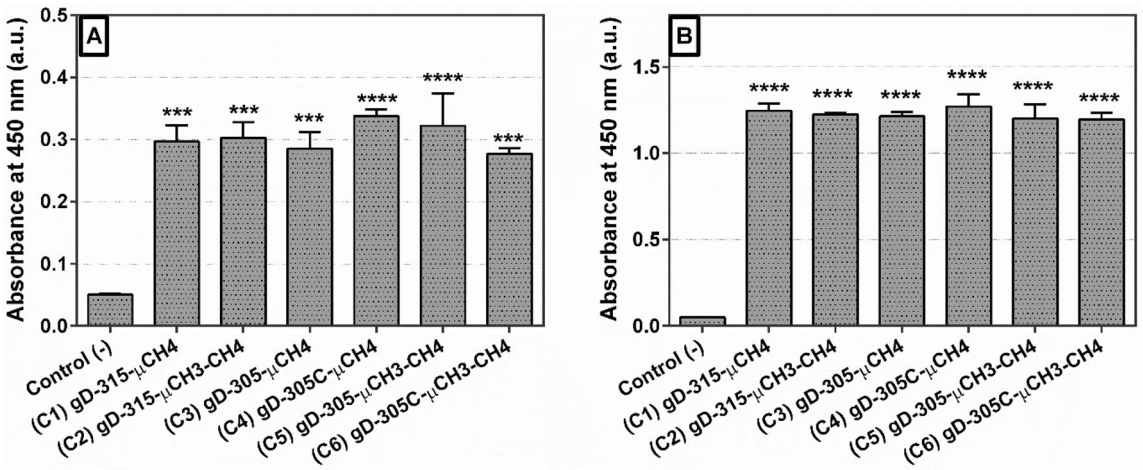

Fig. 3 Histogram representation of ELISA results. Individual vaccine proteins (C1–C6) were coated as antigen in microtiter wells in triplicate and probed by (**a**) anti-gD monoclonal antibody and (**b**) anti-mouse IgM (Fc) antibody, by ELISA

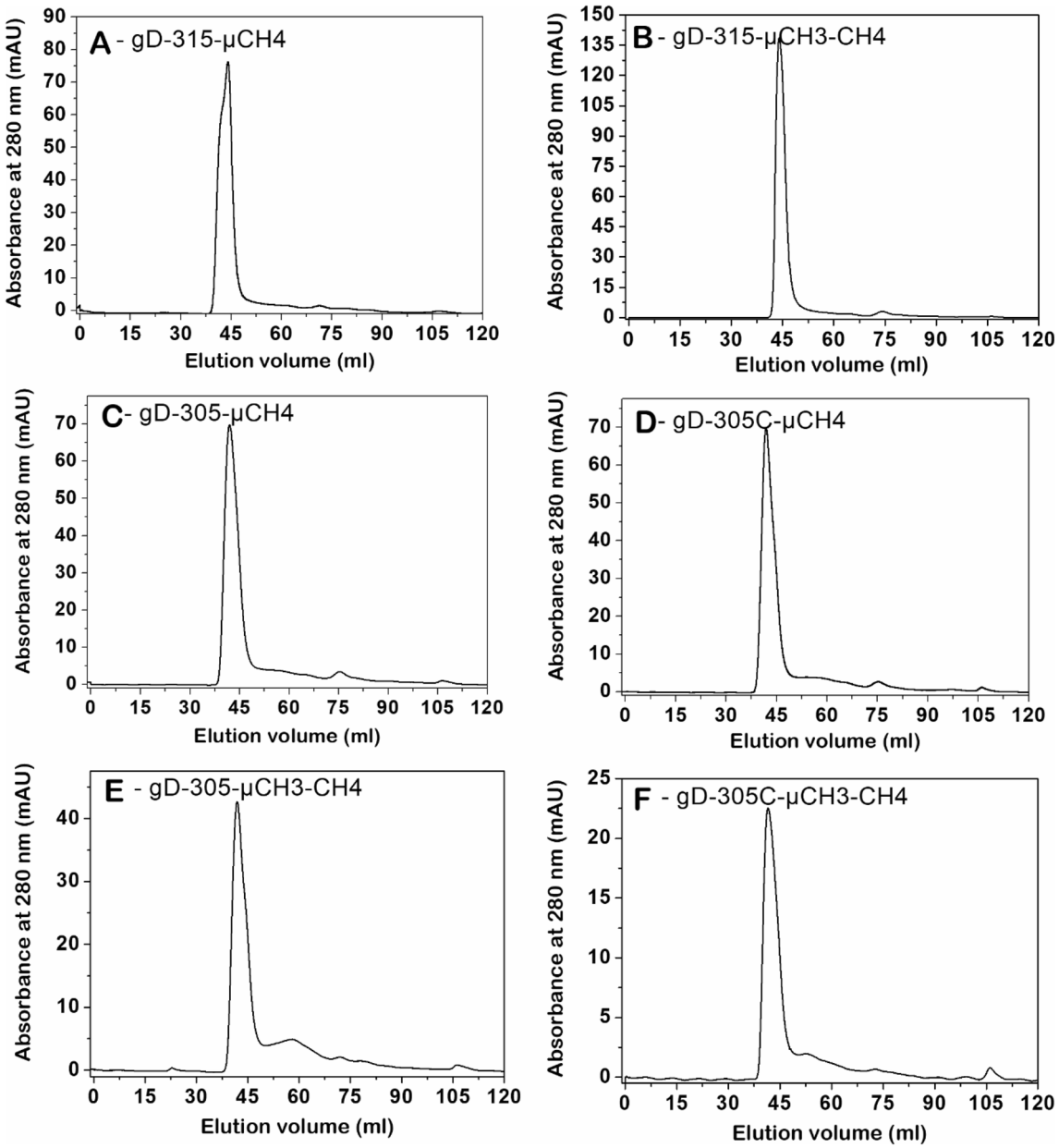

Fig. 4 Analysis of the chromatograms of size exclusion chromatography (SEC), showing the elution profiles of the vaccine proteins (C1–C6), **a** C1- gD-315-μCH4, **b** C2- gD-315-μCH3-CH4, **c** C3- gD-305-μCH4, **d** C4- gD-305C-μCH4, **e** C5- gD-305-μCH3-CH4, and **f** C6- gD-305C-μCH3-CH4. SEC was performed with all the candidate proteins post their in vitro refolding to segregate the vaccine oligomers from that of their unassociated, monomeric and truncated forms

variance (ANOVA) and was found statistically significant ($P < 0.05$) as shown in Fig. 3a. The crystal structure of the gD and mAb E317 complex (PDB: 3w9e), revealed that the E317 mAb is directed against a structural and neutralizing epitope on the surface of gD (Lee et al. 2013). Therefore binding of E317 mAb with all the refolded vaccine proteins suggests their proper refolding and exposure of the antigenic epitopes on the surface of the vaccine oligomers. The binding of anti-IgM antibody to oligomeric vaccine proteins during ELISA also suggests refolding of the Fc domain(s) to their native conformation (Fig. 3b).

Purification of oligomeric vaccine proteins using size exclusion chromatography (SEC)

The SDS-PAGE analysis of refolded vaccine samples showed the presence of both, the full length and truncated vaccine proteins (Fig. 2e). A significant size disparity was

introduced between the monomeric and multimeric forms of the vaccine proteins post refolding; therefore refolded samples of all vaccine candidates were subjected to size exclusion chromatography to further purify the vaccine oligomers. The candidate oligomers (C1-C6) were eluted in a single fraction corresponding to molecular weights in the range of 900–1200 kDa (Fig. 4 and Table 4). The SEC eluted samples of all candidate proteins were analyzed by native PAGE (Fig. 2f) and SDS-PAGE under non-reducing (Fig. 2g), and reducing (Fig. 2h) conditions. As shown in Fig. 2f and g, retention of vaccine proteins in the wells of native and SDS-PAGE gels reconfirms their oligomeric status. The purity of SEC eluted samples (> 95%) showed marked increase as no visible traces of monomeric forms were observed during PAGE analysis (Fig. 2f and g). On the other hand, SDS-PAGE analysis of the same samples under reducing conditions demonstrated reappearance of truncated vaccine proteins in addition to the full-length counterparts (Fig. 2h). This clearly suggests the participation of truncated vaccine proteins in the oligomerization process. The stoichiometry of each vaccine oligomer was determined by comparing its estimated molecular weight with that of its monomeric subunit. Vaccine constructs C1, C5 and C6 assembled with similar stoichiometries of ~ 19 units (18.55, 18.55 and 19.08, respectively). The non-integer stoichiometry obtained with all vaccine candidates may be attributed to the presence of truncated vaccine subunits in the oligomeric complexes. Hence a stoichiometry of ~ 19, theoretically may be assumed equivalent to 20 vaccine subunits wherein two pentameric forms (consisting of 10 subunits each) may have combined together to form a dimer of a pentamer. Since gD is known to exist as a dimer or oligomer in nature, the association of vaccine proteins, in higher than IgM stoichiometry, may be a result of the gD driven phenomenon. Similarly, vaccine constructs C3 and C4 which displayed a stoichiometry of ~ 23 units may have assembled as a dimer of a hexamer, as IgM molecules in absence of J-chain are also reported to exist in hexameric form. The construct C2, largest in size among the rest, was observed to associate with a unique stoichiometry of 15 monomers. Since the stoichiometry in this case, was greater than 10 (pentameric assembly) and 12 (hexameric assembly), the C2 complex was also hypothesized to be an assembly of 20 subunits however not of all full-length vaccine subunits. Therefore, all six vaccine-constructs demonstrated oligomerization with higher stoichiometries (Table 4).

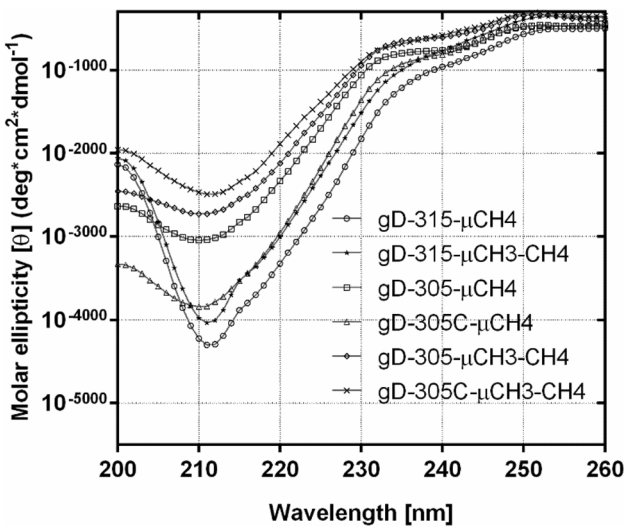

Fig. 5 Representation of the far-UV CD-spectra profiles of the purified vaccine oligomers in the form of a graph wherein the molar ellipticity presented on x-axis and wavelength on the y-axis. The in vitro refolded and SEC purified samples of all the vaccine proteins were subjected to CD analysis

Circular dichroism (CD) spectroscopy and Dynamic Light Scattering of purified oligomeric candidates

The conformation of the secondary-structure elements (α-helix and β-sheet content) of all the vaccine oligomers was ascertained by far-UV CD spectroscopy. The CD spectra depicted as molar ellipticity in Fig. 5, suggested β-sheet to be the dominant secondary-element of all the vaccine oligomers. As per the analysis of CD data using CAPITO software, all constructs were estimated to contain approximately 4% α-helix and 33% β-sheet component (Table 4). Aa per an earlier study the HSV-2 gD was reported to consist of approximately 12% α-helix and 19% β-sheet secondary-elements (Lee et al. 2013); whereas a typical immunoglobulin (IgG) molecule consists of 45–50% β-sheet content (Harris et al. 1997). Therefore the overall estimated percentage of α-helices and β-sheets of the candidates were comparable with the secondary structure composition of gD and an Ig molecule. Hence the CD data supported the earlier ELISA results that suggested proper refolding of the candidate proteins. The higher stoichiometry of oligomeric complexes, indicated their particle-like assembly and therefore the Z-Average size or average-particle-size diameter of individual candidate was determined using dynamic light scattering. The hydrodynamic radius (Rh) of all the oligomers was observed to be ≤ 103 nm; whereas the polydispersity index (PdI) of the oligomers indicated the range of the size distribution of oligomers (SI, Fig. S2 and Table 4). Among all, C2 showed the smallest hydrodynamic radius of 38.2 nm and the lowest polydispersity index of 0.195, suggesting a compact particular assembly of the refolded molecules, with an almost similar size distribution among the entire population of the molecules in the sample. Therefore C2 despite showing lowest stoichiometry of assembly, carrying the gD and Fc subunits of longest lengths, produced the

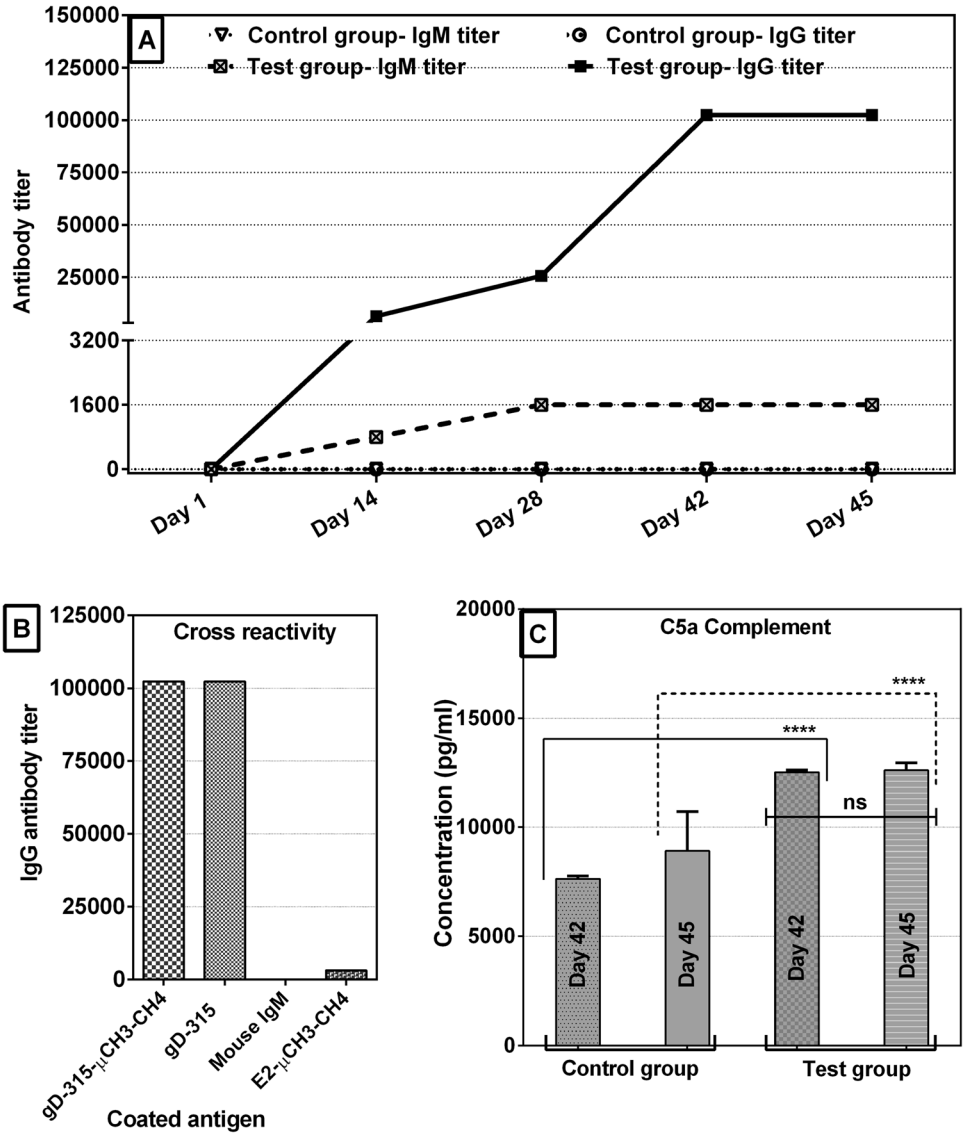

Fig. 6 **a** Analysis of the IgG and IgM antibody titers generated in control group A and test group B mice, which were immunized with vaccine antigen gD-315-μCH3-CH4 (C2), using ELISA. **b** Cross-reactivity ELISA of anti-C2 sera against coated proteins viz. gD ectodomain without IgM Fc domains, mouse IgM antibody and an unrelated antigen ChikV-E2-μCH3-CH4 (domain A of E2 of Chikungunya virus, fused with IgM Fc domains). **c** The levels of C5a complement protein in the serum of the control and test groups collected on day 42 and 45 of the immunization schedule, as determined by ELISA

most compact and thereby stable oligomer and hence it was chosen for further immunological analysis of the complex.

IgG and IgM antibody titer

Since the vaccine candidates were produced using bacterial expression system, their purified samples were tested for determining the endotoxin levels using LAL assay. As shown in SI Fig. S3, all the candidates showed endotoxin levels < 4 Endotoxin units/ml (EU/ml), which is within the permissible limits (< 20 EU/ml) recommended for immunization of model animals using subunit vaccines (Brito and Singh 2011). The candidate C2 oligomers were used to immunize C57BL/6 mice as described in methods section. In order to analyze the anti-gD antibody titer (reciprocal of serum dilution) generated by the mice, sera samples were collected by tail bleeding of mice every 14 days post immunization and were subjected to ELISA for detecting anti-gD IgG and IgM isotype antibodies. The IgM antibody titer was found to be maintained at 1600 till day 45 (Fig. 6a). Fluctuations in the levels of IgM could not be studied as the serum sample collection was performed every two weeks whereas the average response time of IgM release post a stimulus is one week. The IgG antibody titer of 6400 was achieved after primary immunization on day 14 which further showed marginal rise to a value of 12,800 on day 28 post first booster dose and finally peaked to a value of 102,400 on day 42 and remained constant till day 45 (Fig. 6a). A recent study of HSV-2 gD2 vaccine in guinea pigs showed antibody titer generation up to 25,000 and demonstrated strong correlation between antibody titer and protection against genital infection (Hook et al. 2018).

Furthermore, to rule out the possibility of humoral response being directed against the μCH3-CH4 Fc-fragment

instead of the viral antigen, the immune serum was tested to bind other control proteins to check its cross reactivity. As shown in Fig. 6b, in addition to showing binding to its cognate antigen, the anti-C2 sera showed equivalent binding to a control oligomeric gD protein (gD-315) devoid of Fc domains. On the other hand, anti-C2 sera did not show binding to another heterologous control protein with similar design, E2-μCH3-CH4 (containing envelop protein-2 of Chikungunya virus) suggesting the humoral response to be directed against gD (antigen) and not against μCH3-CH4 Fc fragment. Additionally, non-reactivity of anti-C2 serum with native mouse IgM protein further supports the observation that the immune response is gD driven (Fig. 6b). The gD-315 and E2-μCH3-CH4 control proteins used in cross-reactivity assay were expressed in *E. coli* and in vitro refolded to the oligomeric state (data not shown).

Complement activation

Activation of complement system by a vaccine has been shown to help in protection against protozoan and bacterial infections (Beltran-Beck et al. 2014; Behet et al. 2018). In in vivo conditions, the C3 component of complement is required for the induction and maintenance of memory cells of the B cell lineage (Klaus and Humphrey 1986). Additionally, a protective role of complement activation has been shown in various viral diseases like human immunodeficiency virus (HIV), severe acute respiratory syndrome coronavirus (SARS-CoV), Ebola virus, Dengue virus (DENV) and West Nile virus (WNV) (Stoermer and Morrison 2011). The levels of C5a (component of complement system) were determined in the serum samples of the control and test group mice, collected on day 42 and 45 (4th day post third booster immunization by C2). Significantly elevated levels of C5a were observed in the sera-samples of the immunized group mice in comparison to the control group samples, as shown in Fig. 6b. This data further confirms the potential of the vaccine candidate C2 as a strong immune stimulator.

Th1/Th2 cytokine profile

The splenocytes, isolated from the mice immunized with candidate C2 (gD-μCH3-CH4), were cultured for 72 h in vitro under the stimulation of candidate protein at a concentration of 20 μg/ml (endotoxin concentration 0.08 EU/ml) and the cytokine profile of the cultured cells was assessed at 36 and 72 h by ELISA. The cytokine profile of the test group splenocytes, stimulated by C2 candidate, showed a strong bias towards pro-inflammatory response. As shown in Fig. 7a–d, C2 stimulated release of pro-inflammatory (Th1) cytokines viz. IL-1α, IL-2, TNF-α, and IFN-γ, in significant amounts. On the contrary, in case of anti-inflammatory (Th2) cytokines, the levels of TGF-β were significantly suppressed in splenocytes of C2 immunized group up to 72 h, in comparison to the control group. Furthermore, apart from stimulating release of IL-10, C2 did not stimulate the release of two other main Th2 cytokines viz. IL-4 and IL-13, further suggesting the stimulation of a biased Th1 response in addition to stimulating a robust humoral immune response against immunogen gD. Stimulation of a strong, biased Th1 response by the candidate C2-oligomer, demonstrated its virus-neutralizing potential which in combination with a high-titer humoral response may prove effective against viral-replication and infectivity.

Discussion

Approximately half a million people across globe are infected with HSV-2, which is the causative agent of genital herpes and is a risk factor for acquiring HIV-1 infection. Development of a vaccine for prevention of genital herpes is therefore of high priority. Since 1970s various subunit vaccine candidates have been tried and newer candidates with novel modifications have been generated to improve the immunogenicity of the antigens. For example, liposomal formulation of gD ectodomain with adjuvant monophosphoryl lipid A showed protection against HSV-2 in both male and female mice (Olson et al. 2009). In another study, a divalent formulation consisting of gC and gD was used which showed improved protection of dorsal root ganglia in mice and reduction in the frequency of vaginal shedding of HSV-2 DNA in guinea pigs, in comparison to gD alone formulation (Awasthi et al. 2011). Candidates gD and gB, alone and in combination were tested with CpG adjuvant in mice and guinea pigs. The divalent formulation of glycoproteins lead to increased immune response in comparison to the single protein formulations, however the increased immune response that was observed did not correlate to improved protection (Khodai et al. 2011). In an another attempt at development of HSV-2 vaccine, mature form of gG alone was used with and without CpG adjuvant in mice where gG and CpG combined formulation showed low disease scores and higher survival rates in comparison to gG alone formulation, emphasizing the requirement of adjuvants to enhance the immunogenicity of the candidates (Görander et al. 2012). A fusion candidate, where gD antigen was fused with Fc region of IgG, showed neonatal Fc receptor-mediated transport of the fusion vaccine across the mucosal surface and complete protection in the wild type mice; however, the protection lasted for six months only (Ye et al. 2011). In addition to that, scientists are working with peptide vaccine molecules as well. These peptides, often targeted at T-cell and B-cell epitopes, have shown promise in the mouse model (Mo et al. 2011; Wald et al. 2011). In 2017, a trivalent subunit antigen glycoprotein vaccine, consisting of gC, gD and

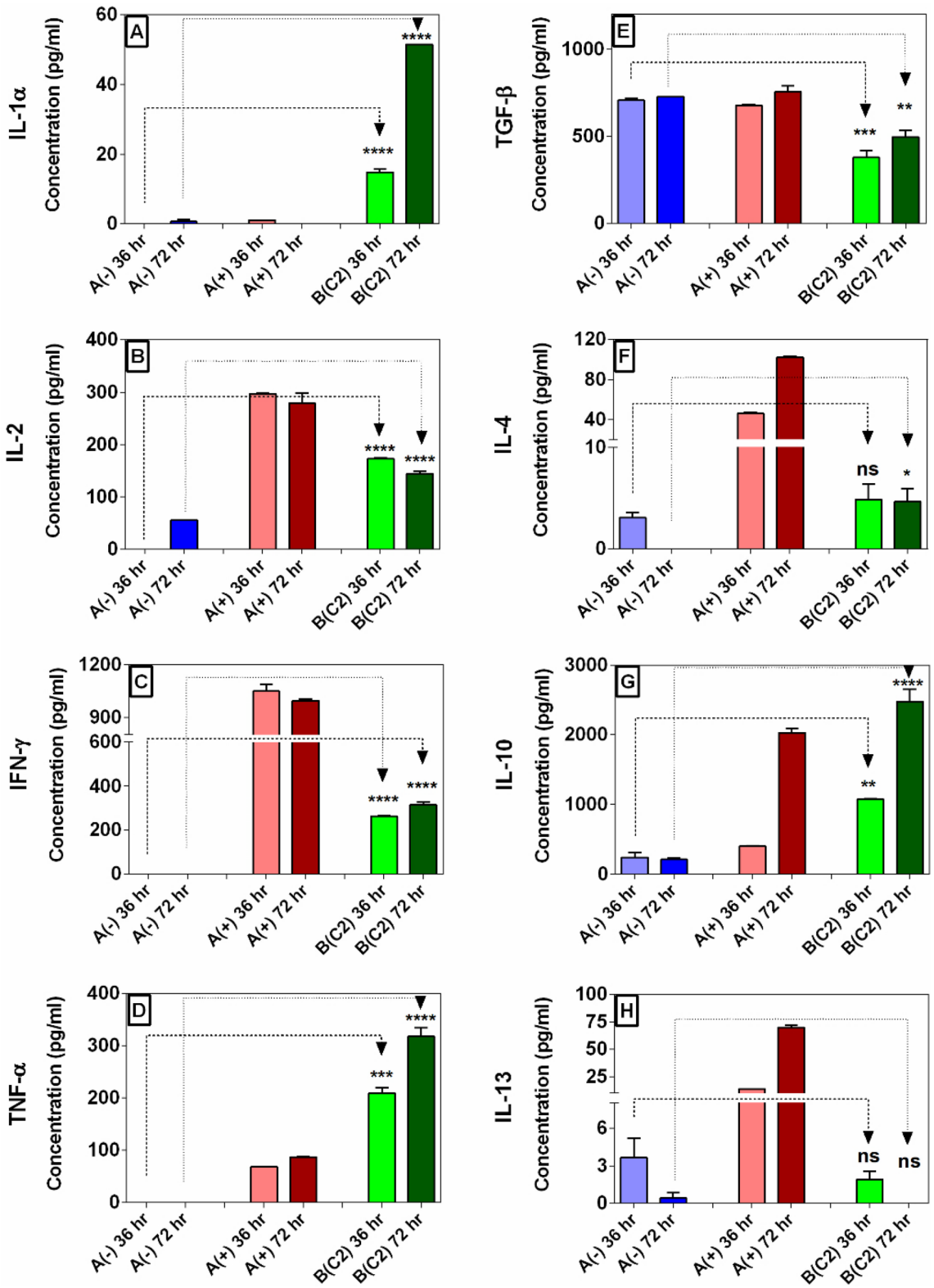

Fig. 7 Analysis of the cytokine profiles of in vitro cultured splenocytes isolated from immunized and control mice splenocytes, **a** IL-1α, **b** IL-2, **c** IFN-γ, **d** TNF-α, **e** TGF-β, **f** IL-4, **g** IL-10, **h** IL-13. The negative control with no treatment is denoted by a negative (−) sign and the positive control with 5 µg/ml Concanavalin A treatment is denoted by a positive (+) sign. In splenocytes of control group mice (non-immunized) were treated with negative and positive controls, whereas the splenocytes isolated from the immunized group B, were treated with the test antigen C2 (gD-315-µCH3-CH4)

gE, was tried as an immunotherapy for genital herpes in the guinea pig genital-infection-model wherein the vaccine formulation reduced the frequency of recurrent genital lesions and vaginal shedding of HSV-2 DNA by approximately 50% (Awasthi et al. 2017). Recently a study used the ribonucleotide reductase 2 (RR2) protein of HSV-2 as a vaccine candidate and showed significant protection against recurrent genital herpes (Srivastava et al. 2019). Since subunit vaccines are safe prophylactic agents with minimal side effects and ease of production, despite their low immunogenicity, myriad attempts have been made and continuing, to develop novel candidates with improved immunogenicity. In the current study, we attempted designing and developing gD ectodomain as an oligomeric vaccine candidate to enhance its immunogenicity. The IgM domains were introduced in the vaccine design to oligomerize gD into decameric or dodecameric complexes. In the absence of J chain, IgM heavy chain domains have been demonstrated to exist as hexamers (Sørensen et al. 2000). Furthermore, the discovery of IgM Fc receptor (FcμR) and their expression on various immune cells had revealed that IgM molecules, in addition to stimulating complement system, may influence a developing immune response through its FcμR mediated effector functions. In humans, FcμR is expressed predominantly by B, T and NK cells (Kubagawa et al. 2009) whereas in mouse, it is predominantly expressed by B lymphocytes (Shima et al. 2010; Ouchida et al. 2012; Liu et al. 2018) and has also been reported to be present on the monocytes, macrophages, granulocytes, and dendritic cells (Lang et al. 2013; Brenner et al. 2014). The FcμR has also been shown to be expressed by B-cells expressing IgG and IgA antibodies indicating its role in heavy chain class switching (Honjo et al. 2012). During early phase of an immune response, B cell are shown to be stimulated by the IgM-FcμR mediated positive signals (Ouchida et al. 2012). Recently FcμR expression on B cells was linked directly to anti-viral IgG responses, where FcμR knockout mice showed lack of robust clonal expansion of B cells and development of fewer IgG plasma cells and memory B cell in the spleen and bone marrow (Nguyen et al. 2017). The above mentioned and other related data accumulated over the last decade has suggested that FcμR may be a central player in B-cell activation and homeostasis (Liu et al. 2018). Furthermore, in addition to binding FcμR, IgM molecules also bind other surface receptors e.g. Fcα/μ receptor (Fcα/μR) and polymeric immunoglobulin receptor (pIgR) (Shibuya and Honda 2006; Honda et al. 2016). The Fcα/μR is expressed by B cells, macrophages, intestinal lamina propria and some other cell types (Shibuya and Honda 2015), whereas the pIgR is mainly expressed on the intestinal epithelial cells (Schneeman et al. 2005; Stadtmueller et al. 2016). Hence by correlating all these reported effector functions of IgM, we hypothesized that the interaction of Fc (antigen fused) with FcμR may stimulate the respective immune cells directly and facilitate efficient uptake and presentation of the chimeric vaccine molecules by these cells.

The current members of the vaccine constructs were designed to be mouse-specific and therefore the IgM Fc regions of mouse origin were used in the study. In about 369-residues long gD molecule, first 316 residues constitute the ectodomain while 317–369 residues constitute the transmembrane domain (Krummenacher et al. 2005). A DNA fragment, encoding full-length ectodomain (1–315 residues) of gD was chemically synthesized and used in vaccine development. As per an earlier report, shorter gD (gD-306 and gD-306_{307C}) molecules of HSV-1 were shown to dimerize and crystalize with ease in comparison to longer gD (Carfí et al. 2001). Therefore, two different lengths (315 and 305 residues) of gD were used in vaccine constructs to enable their dimerization and oligomerization. In case of IgM, Fc regions containing either μCH4 or μCH3-CH4 domains have been reported to attain pentameric as well as hexameric forms; therefore, these two different lengths of IgM Fc region were used for oligomerization of vaccine proteins.

The most stable candidate, C2 (gD-CH3-CH4), was used to immunize mice to assess the humoral and cell-mediated immune response against the gD. The candidate elicited a strong humoral immune response as shown by the generation of high titer (titer of 102,400) anti-gD antibodies. The overall immune response generated by the immunized C57BL/6 mice was further evaluated by culturing splenocytes in vitro and determining their cytokine profile. The immune responses elicited by C2 oligomer attained high titer IgG levels on day 42, post second booster. The antibody titer data determined on day 0, 14, 28, 42 and 45 indicated Fc mediated suppression of IgG response for almost four weeks post-immunization and only reaching peak titer on day 42; as under similar conditions a gD oligomer (without IgM Fc fusion) showed IgG titer peak on day 28 (data not shown). This peculiar IgG response may be attributed to the direct stimulation of B cells via FcμR, first by the C2 candidate molecules and later by the released IgM antibodies. The IgM titer was not followed at an interval of a week, which is its response/release time post-immunization, as the blood/sera collection was performed at an interval of two weeks post respective immunizations. The anti-gD IgM titer, determined every two weeks, showed a minimal constant level of IgM maintained throughout the immunization schedule (Fig. 6a). As per our hypothesis, the suppression and a delayed release of high titer IgG antibodies may have occurred without the help of Th2 cells, which in turn may have allowed stimulation of a biased Th1 response against C2; as was likewise observed in the cytokine profile of cultured splenocytes. On the contrary, the gD oligomer (without IgM Fc) group which achieved high titer IgG response on day 28, followed a more balanced Th1/Th2 response (data not shown). The assessment of the outcome of the above stated immunization

experiments suggest a role of the Fc (fusion candidate) in modifying the immune response against the viral antigen as a molecular adjuvant. Hence the immune response elicited by the C2 fusion candidate in mice showed both the components of an acquired immune response, strong humoral and a biased Th1 cellular response, those may contribute synergistically in neutralizing the virus. The vaccine design has shown promising results in terms of the enhanced humoral and cellular immune response against gD in mice and the efficacy of the fusion candidate may further be evaluated in mice and other model animals by live virus challenge experiments.

Conclusion

In order to enhance the immunogenicity of the HSV-2 antigen, glycoprotein-D, its ectodomain was fused with IgM Fc domains viz. μCH4 and μCH3-CH4. In total, 6 chimeric vaccine-proteins were cloned and expressed in *E. coli* and in vitro refolded into protein oligomers of high stoichiometries of ~ 15–24. Candidate C2 (gD-μCH3-CH4), the most stable oligomer among all, was chosen to immunize the C57BL/6 mice, wherein it elicited a high titer humoral response along with a simultaneous, strong pro-inflammatory Th1 response against gD.

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Author contributions V.K.S. generated the chimeric constructs; V.K.S. and S.T. wrote the manuscript; V.K.S., S.K., R.K.D., A.S.A., N.K.L. and S.T. contributed in the immunization experiments.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval We have complied with all the relevant guidelines and regulations of the Institutional Animal Ethics Committee (IAEC) of the University of Rajasthan (CPCSEA registration no. 1678/GO/RE/S/12/CPCSEA) for animal testing and research. All the experimental protocols performed were approved by IAEC for a study in collaboration with Dr. A. S. Ansari's lab (IAEC approval No: UDZ/IAEC/II/04; dated 10–05-2019) in C57BL/6 strain of mice.

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