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Marked enhancement of the immunogenicity of plant-expressed IgG-Fc fusion proteins by inclusion of cholera toxin non-toxic B subunit within the single polypeptide

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Summary

Immunoglobulin G (IgG)-based fusion proteins have been widely exploited as a potential vaccine delivery platform but in the absence of exogenous adjuvants, the lack of robust immunity remains an obstacle. Here, we report on a key modification that overcomes that obstacle. Thus, we constructed an IgG-Fc vaccine platform for dengue, termed D-PCF, which in addition to a dengue antigen incorporates the cholera toxin non-toxic B subunit (CTB) as a molecular adjuvant, with all three proteins expressed as a single polypeptide. Following expression in *Nicotiana benthamiana* plants, the D-PCF assembled as polymeric structures of similar size to human IgM, a process driven by the pentamerization of CTB. A marked improvement of functional properties *in vitro* and immunogenicity *in vivo* over a previous iteration of the Fc-fusion protein without CTB [1] was demonstrated. These include enhanced antigen presenting cell binding, internalization and activation, complement activation, epithelial cell interactions and ganglioside binding, as well as more efficient polymerization within the expression host. Following immunization of mice with D-PCF by a combination of systemic and mucosal (intranasal) routes, we observed robust systemic and mucosal immune responses, as well as systemic T cell responses, significantly higher than those induced by a related Fc-fusion protein but without CTB. The induced antibodies could bind to the domain III of the dengue virus envelope protein from all four dengue serotypes. Finally, we also demonstrated feasibility of aerosolization of D-PCF as a prerequisite for vaccine delivery by the respiratory route.

Introduction

Dengue is the world's fastest-growing tropical infectious disease. Hundreds of millions of people are affected or at high risk, with South America and South-East Asia being the most affected parts of the world (Bhatt *et al*., 2013). Ordinarily, dengue has mild to moderate clinical manifestations in most people but in a proportion of affected individuals, it can cause severe, life-threatening complications such as hemorrhagic fever and dengue shock syndrome. Only supportive treatment is currently given, based on anti-inflammatory molecules and painkillers. This reduces the symptoms but does not cure the disease and globally, out of approximately 500 000 cases of severe dengue, > 40 000 die, mostly young children. Such loss of life could be prevented if there was an effective vaccine that could prevent infection or the most severe clinical symptoms and death. The recently licensed Sanofi-Pasteur Dengvaxia vaccine is available, but its efficacy is relatively poor (50%) (Hadinegoro *et al*., 2015) and it is not recommended for children under the age of nine. Better efficacy rates in all populations were seen for Takeda's QDENGA vaccine which has been recently approved by the EU, the UK and several dengue endemic countries such as Brazil and Indonesia, but even so, it is far from perfect, as varying levels of protection were observed for different dengue serotypes, and the protection also gradually waned during the extended follow-up period of 54 months (Biswal *et al*., 2019, 2020; Rivera *et al*., 2022; Thomas, 2023). Similar issues are besetting also the attenuated virus tetravalent Butantan vaccine (Kallas *et al*., 2020), the last to complete phase 3 efficacy trials, though their yet unpublished/peer-reviewed results can be only seen on the company's website (Butantan's dengue vaccine has 79.6% efficacy, partial results from 2-year follow-up show - Instituto Butantan). Apart from the above three candidates, there are a number of other both clinical and preclinical dengue vaccine candidates currently in research and development, mostly based on attenuated or inactivated viruses, viral vectors and adjuvanted proteins (reviewed in Prompetchara *et al*. (2020)) but it is clear that more effort has to be put in further building and diversifying the dengue vaccine pipeline.

We have a long-standing interest in molecular engineering and testing of adjuvant-free subunit vaccine candidates that

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autonomously derive their own adjuvanticity. These are based on Immunoglobulin G (IgG) Fc fragments fused to the antigen of interest, that are engineered to polymerize and form immune complexes for enhanced uptake by antigen-presenting cells (APCs). Several iterations of these Fc-fusion proteins have been generated, showing a considerable vaccine potential against infections such as TB and dengue (Kim *et al*., 2015, 2017, 2018; Pepponi *et al*., 2013, 2014; Webster *et al*., 2018). Thus, in the context of dengue, we have previously developed a novel, protein-only based vaccine platform that derives its own adjuvanticity from fusing the antigen to the IgG antibody heavy chain Fc fragment that carries the IgM-tail piece responsible for hexamerization of IgM, thus resulting in polymeric structures which we termed PIGS (Polymeric Immunoglobulin Scaffold) (Kim *et al*., 2017, 2018). Following immunization of mice, this construct induced superior humoral and cellular immune responses including memory and effector T cells, compared to vaccination with antigen alone (Kim *et al*., 2018). However, this approach was not sufficiently robust at inducing immune responses at mucosal sites, most likely because of restricted access of the vaccine to APC, following mucosal delivery. In contrast, the non-toxic CTB fusion with dengue antigen was shown to induce both systemic and mucosal antibodies by oral administration of whole plant cells expressing this construct (Kim *et al*., 2016). The rationale for whole cell approach was that encapsulation of antigen by plant cell wall would protect it from the acidic environment until reaching intestinal tissues (Kim *et al*., 2013; Kurokawa *et al*., 2013). However, while we demonstrated the immunogenicity of that vaccine construct, the formulation itself is not practical to control the antigen dose and may be more suitable for a veterinary vaccine rather than a human application.

Therefore, to overcome the limitation of a restricted number of APC that the vaccine can access following either systemic or mucosal delivery, and to overcome mucosal tolerance mechanisms in the latter case, we have combined the two above approaches, and now generated and tested the latest iteration of the Fc-fusion protein which incorporates a molecular adjuvant, the cholera toxin non-toxic B subunit (CTB), to stimulate influx of APC to the mucosal sites. We believe that this new vaccine platform now 'ticks all the boxes' to be a successful, adjuvant-free mucosal or systemic vaccine. It contains three molecular components fused within a single polypeptide: CTB, the antigen of interest and the IgG-Fc fragment (hence 'PCF' – Platform CTB-Fc). The unique advantage of this vaccine platform is that it does not require exogenous adjuvants, since CTB is a well-known mucosal immune modulator (and applicable to humans - a component of Dukoral - the current oral cholera vaccine), while the polymeric Fc facilitates efficient uptake by infiltrated APCs. The PCF constructs form pentamers (through CTB (Merritt *et al*., 1994)) and thus deliver five copies of antigen to APC with each polypeptide, which further increases due to the dimerization of IgG-Fc and immune complex formation.

In this report, we present the evidence for robust immunogenic properties of this novel adjuvant-free vaccine delivery system *in vitro* and *in vivo* and show that it can be used for the induction of strong systemic and mucosal immunity.

Materials and methods

Generic structure of the PCF vaccine delivery platform

The gene for the human version of PCF structure consisting of CTB and IgG1-Fc was plant codon optimized and synthesized. For the subcloning of PCF into the expression vector, pTRAk.2 (Sack *et al*., 2007), the *NcoI* and *XbaI* restriction enzyme sites were added at the N-terminal sequence of CTB and the C-terminal sequence of IgG1-Fc, respectively. Additional restriction enzyme sites (*BamHI* at the C-terminal sequence of CTB and *SpeI* at the N-terminal sequence of IgG1-Fc) were added for insertion of the target antigen (see next section) between CTB and Fc. To allow flexibility between three vaccine components, the GPGPGS hexapeptide between CTB and antigen, and the Y-shaped partial sequence of the C-terminal CH_1 domain-hinge region of IgG1 constant heavy chain between antigen and the Fc, were added (Figure 1b). To improve the level of expression and polymerization within the host cell, the endoplasmic reticulum (ER) to Golgi trafficking and ER retrieval signal peptide was cloned at the C-terminal side of the PCF molecule.

Dengue PCF (D-PCF) construction and expression in plants

The gene for dengue antigen, consensus envelope domain III, cEDIII, was amplified using Phusion® High-Fidelity DNA polymerase (NEB) with forward, scEDIII-F (5′- ttttggatccaagggcatgtcctac-3′) and reverse, scEDIII-R (5′-ttttactagt gcgagctctcaggagccctt-3′), primers and the plasmid DNA, cEDIII-PIGS (Kim *et al*., 2017) as the template. To generate D-PCF, the cEDIII PCR fragment bearing BamHI and SpeI restriction enzyme sites was then subcloned into pTRAk.2 vector containing PCF gene framework (above), for tobacco expression. Then, D-PCF was transformed into *Agrobacterium* (strain GV3101 containing pMP90RK helper plasmid) by electroporation and selected on YENB medium (7.5 g of Bacto-yeast Extract, 8 g of Nutrient broth, pH 7.5), containing 50 μg/mL each of carbenicillin and rifampicin.

For transient expression in plant cells, the vacuum infiltration method was performed. Briefly, the covered pots with about 6 to 8 weeks old *Nicotiana benthamiana* were submerged in the liquid suspension of *Agrobacterium* which was diluted to OD_{600} = 0.1–0.2 in infiltration medium (10 mM MES, 10 mM $MgCl_2$) and subjected to decreased pressure followed by rapid re-pressurization (Bechtold and Pelletier, 1998). The plants were kept in a short-day light cycle (8 h light/16 h dark; light intensity 80–100 μ mol m^{-2} s^{-1}) at 21 °C during the day and 16 °C during the night until harvesting leaves at 5–7 days after infiltration.

Purification and fractionation of D-PCF

To extract plant-derived D-PCF, the infiltrated leaves subsequently frozen at −70 °C were homogenized in a blender with 2 ~ 3 volumes 0.1 M Tris–HCL buffer (pH 8.0) containing 0.05% of sodium cholate hydrate (Sigma) and the crude extracts filtered through 3-layers Miracloth (Calbiochem) and centrifuged at 13 000 rpm for 1 h in a ROTINA 48R centrifuge (Hettich Zentrifugen). The supernatant was sterilized through a 0.22 μm filter before applying to a protein A agarose affinity column (Sigma). After extensive washing with Tris extraction buffer, the bound protein was eluted in 0.1 M glycine-HCl, pH 2.7, and the fractions neutralized by the addition of 1 M Tris base (pH unadjusted). The eluted fractions were combined and concentrated by ultrafiltration (Amicon® centrifugal filters with a MWCO of 100 kDa, Merck Millipore) followed by dialyzing against PBS buffer at room temperature overnight using a dialyzing cassette with 10 kDa molecular weight cut off (Slide-A-Lyzer, Thermo Scientific, US). The concentration of protein was determined by measuring optical density at 280 nm using

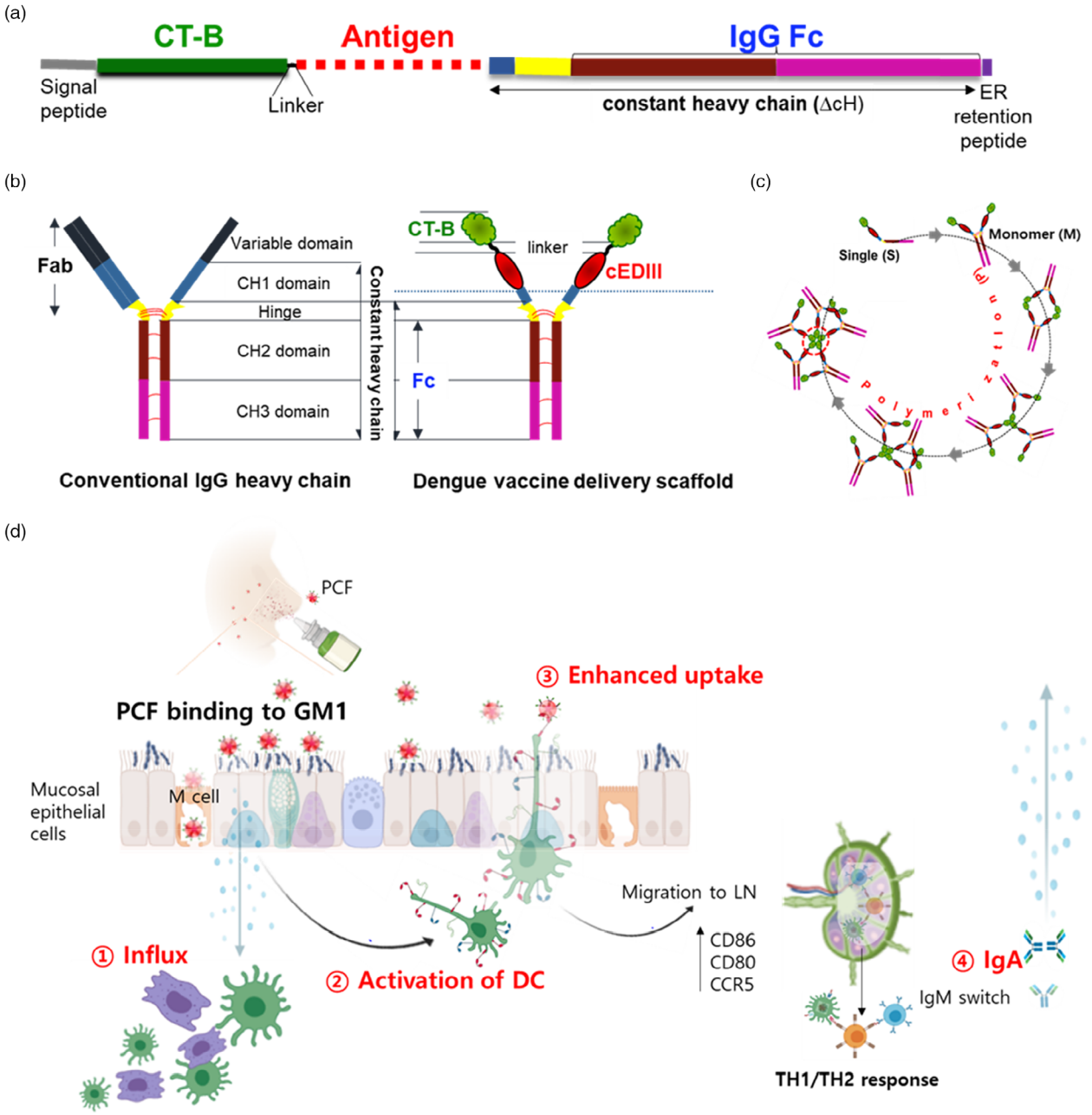

Figure 1 Construction of PCF fusion protein and its proposed mode of action. (a) Genetic construct linear sequence; the three components of PCF vaccine platform are the non-toxic cholera toxin B subunit (CT-B), antigen of interest (here, the consensus domain III of the dengue virus envelope glycoprotein, cEDIII) and truncated constant heavy chain (ΔcH) of human IgG1. The signal sequence (derived from human IgG1) and the ER retention signal peptide (KDEL) were included at the N and C termini, respectively. (b) homo-monomeric structure of vaccine delivery scaffold of D-PCF (right) in comparison to conventional IgG heavy chain (left); (c) schematic representation of a possible mode of polymerization of the D-PCF fusion protein through CTB. (d) Proposed mechanism of adjuvanticity of the PCF vaccine platform; PCF is designed to maximize mucosal adjuvanticity with multiple Ag display and double targeting of both mucosal epithelial and APCs surface, leading to enhanced immune responses.

NanoDrop™ 2000/2000c Spectrophotometer (ThermoScience). Intact D-PCF protein was subjected to size-exclusion chromatography (SEC) on a HiLoad 16/600 Superdex 200 column (GE Healthcare, USA) equilibrated with PBS pH 7.4 and connected to an ÄKTA pure (GE Healthcare, USA) FPLC system. The fractionated D-PCF was collected for further analysis. High-molecular weight (HMw) and low-molecular weight (LMw) fractions were collected separately.

Protein analysis using gel electrophoresis and immunoblotting

To confirm the expression and composition of D-PCF, the protein was purified and the heat-treated samples for 5 min at 96 °C were run on 4%–12% Bis-Tris gels (Life Technologies) using NuPAGE® MOPS SDS running buffers (Life Technologies). Following electrophoresis, gels were stained by Coomassie or

subjected to Western blot analysis with peroxidase-conjugated anti-human IgG antiserum (1:2500 dilution; The Binding Site) for detection of the IgG Fc portion, or with mouse anti-dengue virus monoclonal antibody (1:2500 dilution; Bio-Rad AbD Serotec) followed by anti-mouse IgG (light chain specific) peroxidase-conjugated antiserum (1:2500, Jackson ImmunoResearch), or with rabbit anti-CT polyclonal antibody (1:2500 dilution; Sigma) followed by anti-rabbit IgG peroxidase-conjugated antiserum (1:2500, Sigma). The washed blots were developed using the ECL Plus Western blotting detection system (GE Healthcare).

Size measurement of polymeric D-PCF molecule by Zetasizer

Hydrodynamic diameter of proteins was determined by dynamic light scattering (DLS). Various concentrations (0.2 ~ 4 mg/mL) of proteins including unfractionated or fractionated D-PCF, commercial IgG (Millipore) and serum IgM (Invitrogen) were prepared in 50 μL of PBS and applied into the disposable UV micro cuvette (0.85 mL, Brand) for Zetasizer Nano-ZS instrument (Malvern) measurements. The particle distribution was analysed by measuring intensity and mass, and the average Zeta-size of the molecules.

ELISA for GM1 ganglioside and complement C1q binding

To confirm binding of D-PCF to GM1 (5 μg/mL of monosialoganglioside GM1, Sigma) or complement C1q protein (5 μg/mL of human C1q (Calbiochem)) was coated onto ELISA plates in PBS buffer (pH 7.4) and incubated overnight at 4 °C. After blocking in 5% non-fat dry milk protein solution in PBS, 2-fold serial dilutions of samples in triplicates were added and incubated at 37 °C for 2 h. The PBS buffer alone or commercial human IgG antibody (Sigma) was used as the negative control. For GM1 binding (via CTB), peroxidase-conjugated anti-rabbit IgG antiserum (Sigma) was used, followed by anti-CT polyserum (Sigma) at 1/5000 dilution. For binding to C1q protein (via Fc), peroxidase-conjugated anti-human IgG antiserum (The Binding Site) was used at 1/2500. The peroxidase reaction was developed by adding 50 μL of TMB substrate solution (Bethyl Laboratories, Inc) to each well. The colour reaction was stopped by the addition of 25 μL/well of 2 M H_2SO_4 and the absorbance was determined at 450 nm using a Sunrise plate reader (Tecan, UK).

Binding of D-PCF to APC surface by flow cytometry and internalization by confocal microscopy

To test the capacity of D-PCF to bind to Fcγ-receptor bearing cells, THP-1 monocyte/macrophage cell line (ATCC) was grown in RPMI medium supplemented with 10% Foetal Bovine Serum (FBS); 1 million cells were suspended in 100 μL 3% BSA in PBS buffer and incubated on ice for 2 h with 5 or 20 μg/mL of D-PCF or D-PIGS, used as comparator. Unbound protein was removed by washing 2 times in binding buffer and 7.5 μL of secondary antibody anti-human IgG-FITC antiserum (The Binding Site) was added, followed by incubation for a further 1 h on ice. After washing as before, cells were resuspended in 500 μL of binding buffer and analysed for green fluorescence in a Becton–Dickinson flow cytometer. A secondary antibody alone was used for background staining. The data was analysed with the FlowJo v10 software program.

To visualize antigen uptake by APCs, 200 000 THP1 cells in 96-well flat bottom plates (100 μL/well) were prepared overnight. 5 μg/mL of the fractionated D-PCF in 50 μL/well was added and cells were incubated in the CO_2 incubator at 37 °C for 5 h. To permeabilize the cells, 50 μL of the IC fixation buffer (Invitrogen) was added to each well for 15 min at 4 °C. The cells were then washed three times with 1× permeabilization buffer (Invitrogen) followed by staining with 1:100 dilution of anti-human IgG-FITC (Biolegend) for 45 min at 4 °C. The cells were washed twice with permeabilization buffer and DAPI nuclear stain (ThermoFisher) added (1:1000) for 5 minutes. The cells were washed twice and reconstituted in 100 μL permeabilization buffer and transferred to black plates for the fluorescent image analysis using a Nikon A1R confocal microscope (Nikon).

moDC differentiation and activation

CD14+ monocytes were enriched from PBMCs using the MojoSortTM Human Pan Monocyte isolation kit (BioLegend) according to the manufacturer's recommendations. Monocyte isolation yielded >90% purity and > 95% viability of CD14+ monocytes (data not shown). Right after isolation, enriched monocyte fractions were plated in 24-well tissue culture plates (Corning Inc.) at a density of 1×10^6 cells per well. Complete RPMI 1640 media supplemented with 70 ng/mL granulocyte-macrophage colony-stimulating factor (GM–CSF) (Peprotech) and 50 ng/mL interleukin-4 (IL-4) (BioLegend) was used for 7 days to induce differentiation of monocytes to monocyte-derived dendritic cells (moDCs). Fresh differentiation media was added to wells every 2 days. At day 7, cells were detached from wells using an enzyme-free dissociation solution (Merck) and replated at a density of 5×10^4 cells/well in 96-well U bottom tissue culture plates (Corning Inc.) in complete RPMI-1640. Cells were subsequently treated with either lipopolysaccharide (LPS) (100 ng/mL) (Sigma), human IgG (Sigma, 10 μg/mL), unfractionated, HMw or LMw D-PCF (10 μg/mL). At day 9, cells were harvested to measure activation-induced cell-surface markers (MHC-I/HLA-ABC, MHC-II/HLA-DR, CD80, CD86 and PD-L1) by flow cytometry. Briefly, wells were washed with DPBS (Sigma) and then incubated for 45 min at 4 °C with 1:500 eBioscienceTM fixable viability dye eFluor780TM (Invitrogen), 1:250 Human TruStain FcXTM (BioLegend) 1:200 APC anti-human HLA-DR (BioLegend), 1:200 PE anti-human HLA-A,B,C (BioLegend), 1:200 PE/Cyanine 7 anti-human CD86 (BioLegend), 1:200 Brilliant Violet 510TM anti-human CD80 (BioLegend) and 1:200 Brilliant Violet 42TM anti-human PD-L1 (BioLegend) in DPBS. After incubation, cells were washed and resuspended in DPBS. Cell acquisition was done using CytoFLEX S (Beckman Coulter) flow cytometer. FlowJoTM v10.8.1 (TreeStar) was used for analysis of FACS data.

THP-1 and moDC internalization assays

THP-1 or moDCs were plated in 96-well U bottom plates at a density of 5×10^4 cells/well. Cells were then treated with either D-PCF (10 μg/mL) or D-PIGS (10 μg/mL) and incubated for 12 h in a humidified incubator set to 37 °C and 5% CO_2. Afterwards, cells were washed with PBS and stained for 20 min with 1:500 eBioscienceTM fixable viability dye eFluor780TM (Invitrogen, 65-0865-14). Cells were subsequently fixed and permeabilized using the IC fixation buffer (Invitrogen, 00-8222-49) for 15 min, followed by intracellular staining with 1:100 FITC anti-human IgG Fc (Biolegend®, Clone M1310G05, 410 720) for 45 min. Stained cells were acquired using a CytoFLEX S flow cytometer (Beckman-Coulter) and analysed using FlowJoTM v10.8.1 (BD Life Sciences, Ashland, OR). Data were expressed as mean fluorescence intensity (MFI). For imaging experiments, cells were seeded at 1×10^5 cells/well in 96 black well plates (Corning Inc., 3603).

Cells were left to attach for 2 h, then treated with either monomeric IgG (10 μg/mL), D-PCF (10 μg/mL) or D-PIGS (10 μg/mL) for 12 h. Wells were washed with PBS, followed by fixation and permeabilization using an IC fixation buffer (Invitrogen, 00-8222-49). Afterwards, cells were stained with 1:100 FITC anti-human IgG Fc (Biolegend®, Clone M1310G05, 410 720) and 1:2000 Phalloidin-iFluor 647 (abcam, ab176579) for 45 min. Cells were then stained with 1:1000 DAPI (Thermo Scientific, 62 248) for 5 min. Confocal image acquisition was done using Nikon A1R Microscope and Nikon NIS-Elements C software.

Human alveolar epithelial cells binding and uptake of D-PCF

Primary human alveolar epithelial cells (hAECs) obtained from Generon were maintained in Endothelial cell growth medium-2 (Promocell) at 37 °C in a 5% CO_2 humidified atmosphere. hAECs were seeded at a density of 50 000 cells per well in a 96-well tissue culture plate. Cells were then treated with either human IgG or D-PCF overnight. The following day, cells were harvested for confocal imaging and flow cytometry analysis.

For confocal imaging, hAECs were washed twice with PBS and fixed with Fix/Perm buffer (ThermoFisher Scientific) according to the manufacturer's instructions. Cells were then permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 10 min and stained with phalloidin, DAPI and anti-human IgG-FITC (Biolegend). After washing and resuspension in PBS, cells were imaged using a confocal microscope (Nikon A1R).

For flow cytometry analysis, human IgG or D-PCF-treated cells were washed twice with PBS and stained with eFluor870 fixable viability dye (ThermoFisher Scientific) for 20 min at 4 °C. The cells were then fixed with Fix/Perm buffer for 20 min at 4 °C, permeabilized with 0.1% Triton X-100 for 10 min and stained with anti-human IgG-FITC for 1 h at 4 °C. Flow cytometry acquisition was performed using the Cytoflex (Beckman Coulter) and analysed using FlowJo V10.

Binding avidity of D-PCF to low-affinity FcγRIII by surface plasmon resonance

The avidity of the D-PCF binding to human FcγRIII (CD16a) was measured using a Biacore ×100 instrument (GE Life Sciences, Little Chalfont, UK). Briefly, 12 000 RU of an anti-His antibody was immobilized on both flow channels of a CM5 sensor chip using amine coupling chemistry (His capture kit, GE Life Sciences). For the sample, recombinant human CD16a containing a His tag (1960-FC, R&D Systems) diluted to 2 μg/mL in HBS-EP+ running buffer was captured on flow channel 2 to a level of 750 RU. In total, 500, 166.7, 55.6 nM and 18.5 nM of D-PCF or hIgG samples (as an intermediate control) were injected over both flow channels with a contact time of 80s and a flow rate of 30 μL/min, and dissociation monitored for at least 500 s. Regeneration of the surface was achieved by a 30s pulse of 10 mM glycine pH 1.5.

Immunization of CD64 transgenic mice

For immunization, 12–20 weeks old, inbred male and female FcγRI/CD64 mice were used (van Egmond *et al.*, 1999). The mice were kept under defined environmental conditions, with food and water ad libitum and within an enriched environment, under an established project licence and with approval of the St George's Ethics Committee and the UK Home Office. In a pilot experiment, only D-PCF was used whereas in the main study, a direct comparison of D-PCF and D-PIGS was performed. Three mice per group were immunized subcutaneously with 30 μg of D-PCF or D-PIGS in 100 μL of PBS, with a separate group of mice injected with 100 μL of PBS only. Mice were then further immunized twice with 30 μg of respective vaccines (or PBS) intranasally, at weeks 4 and 6, and were bled after each immunization to monitor the antibody titers. At week 7, mice were sacrificed and bled by cardiac puncture and their bronchoalveolar lavage (BAL) recovered by flashing intratracheally their lungs with 0.5 mL PBS. Also, the spleens were collected for analysis of T cell responses.

Analysis of humoral response by ELISA

To test the vaccine-induced antigen-specific and serotype-specific cross-reactive antibody responses, the immune sera and BAL fluids were analysed by ELISA. ELISA plates were coated with recombinant consensus EDIII (cEDIII) or $EDIII_{1\text{-}4}$ antigen (10 μg/mL) and probed with either 10-fold diluted mouse sera (for endpoint titre measurements) or with 2- or 3-fold serial dilutions of sera or BAL, respectively, for isotype and sub-type mapping. Antigen-specific IgG, IgG1, IgG2a, IgG2b and IgA responses were detected by peroxidase-conjugated sheep anti-mouse secondary antibodies (The Binding Site).

Analysis of cellular responses

To obtain splenocytes, spleens were extracted aseptically from immunized mice, pooled and homogenized in 10 mL of complete RPMI medium (Sigma) using a 5-ml syringe plunger. The tissue was squeezed through a 70 μm cell strainer (BD FalconTM). The released cells were spun, and the pellet was resuspended in RPMI medium. To eliminate red blood cells, the pelleted cells were incubated with ACK lysing buffer (Gibco) for 3 min at 37 °C and washed two times with 25 mL complete medium. Triplicate cultures were seeded into 96 well U-bottom plates at a density of 3×10^5 cells/well, in 200 μL medium and stimulated with 10 μg/mL of dengue antigen or PBS as a control. After incubation of cells for 48 h at 37 °C, 100 μL of supernatant was removed for Th1/Th2 and IL-17 cytokine ELISA assays. The experimental procedure described by the manufacturer (Mouse Th1/Th2 and Th17 ELISA Ready-SET-Go kit; affymetrix eBioscience, USA) was then followed.

Aerosolization of D-PCF

We tested the feasibility of D-PCF aerosolization and protein recovery, as a prerequisite for human application by respiratory route. In total, 2 mL of D-PCF formulated in PBS/0.1% Polysorbate-80 buffer were loaded into OMRON MicroAIR nebulizer (U22, Japan) as described in Tran et al., (Tran *et al.*, 2020) and following 1 min aerosolization, condensate collected in a glass cylinder and subsequently analysed for protein content, C1q, GM1 and APC binding, as described above.

Statistical analysis

The ELISAs for GM1, C1q binding, antibody detection and cytokine measurements were performed in triplicates and the values were shown as the mean +/− standard deviation. Imaging and quantitative analysis by confocal microscopy were performed on four representative 0.5 mm^2 square fields that contained a minimum of 20 cells or more. The statistically significant differences between multiple test groups ($P < 0.05$) in various assays were determined by One-way ANOVA followed by Tukey's multiple comparisons correction test comparing, D-PIGS and D-PCF.

Results

Construction of D-PCF and proposed mechanisms of polymerization and mucosal adjuvanticity

We previously described an IgG-Fc fusion strategy for efficient delivery of dengue cEDIII antigen to APCs and demonstrated its immunogenicity and vaccine potential (Kim *et al.*, 2017, 2018). To overcome the limitation of a restricted number of APC that the vaccine can target when delivered either systemically or mucosally, we further modified this concept by the addition of the molecular adjuvant CTB resulting in a 3-component single PCF construct (Figure 1a), which includes CTB, IgG-Fc and the dengue antigen. For structural flexibility of the polypeptide, the general hexapeptide linker, (GPGPGS) between CTB and antigen, and human IgG1-CH1 fragment (last 10 amino acids of the IgG-CH1 domain, corresponding to the final β-strand), between antigen and IgG-Fc, were added, so that the putative monomer forms a Y-shape typical of IgG, including the hinge region (Figure 1b). The ER retention peptides at the C-terminus of PCF (Figure 1a) were included to increase its retention and maximize the expression and assembly within the ER of the plant cells. The D-PCF can undergo polymerization through CTB, potentially forming pentamers (as native CTB does) and possibly other polymeric forms, as schematically indicated in Figure 1c. The added value of CTB is schematically shown in Figure 1d, whereby in the case of mucosal delivery, CTB interacts with mucosal ganglioside 1 (GM1), causing an influx of submucosal APC, which then targets D-PCF binding via the Fc component, which in turn leads to induction of immune responses, including mucosal IgA secretion.

Expression and size-characterization of D-PCF molecule

The expression of D-PCF fusion protein in *Nicotiana Benthamiana* plants was verified by denaturing electrophoresis, Coomassie staining and immunoblotting with each of the three component-specific antibodies (Figure 2a). Partially reassembled polymers under these denaturing conditions showing higher molecular weights (expected size 208 kDa for homodimer to 520 kDa for pentamer), as well as the monomers (expected 104 kDa) and single chain constructs (expected 51 kDa), could be observed (Figure 2a). The molecular weights (Mw) are computably calculated by ProtParam in Expasy (https://web.expasy.org/protparam/) without two putative *N*-glycosylation sites. The Mw of purified D-PCF fusion protein was verified by non-denaturing SEC analysis and the results showed that it consisted of 92% polymer, 3.7% monomer and 1.8% of lower Mw species, with polymeric fraction somewhat bigger than pentameric human IgM, and monomeric fraction comparable to IgG (Figure 2b). These estimates were broadly corroborated with the Zetasizer measurements, which showed an average size of polymeric D-PCF (96.4 nm) as greater than that for pentameric IgM (52.6 nm) and IgG (11.9 nm) (Figure 3c, left panel). Further analysis of size distribution by intensity revealed that most of D-PCF existed as polymers reflecting light in the same range as pentameric IgM, although a smaller non-pentameric fraction of IgM not detected by SEC appeared to disproportionately reflect the light intensively (Figure 3c, right panel). Taken together, these analyses showed that the presence of all three D-PCF components could be verified and that the plant-expressed polypeptide is predominantly a high molecular weight (HMw) form similar in size to human pentameric IgM. Furthermore, we demonstrated that D-PCF could be efficiently purified using protein A/G affinity chromatography, with yields in this laboratory scale being approximately 20 mg/1 kg of fresh weight leaf tissue.

Fractionation and properties of HMw and LMw D-PCF

Since we also observed the presence of a low Mw fraction in unfractionated D-PCF, we wanted to compare the biophysical and functional properties of the HMw and LMw fraction, to establish the added value of polymerization. Thus, D-PCF was

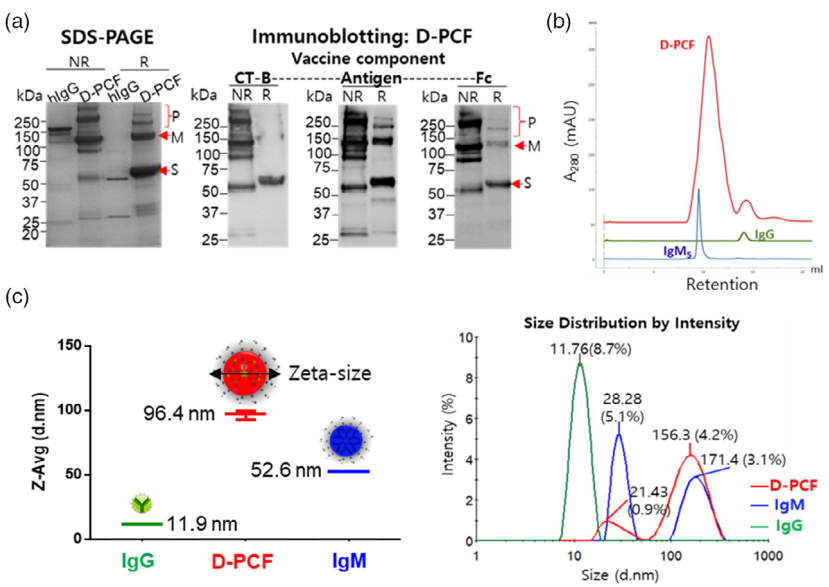

Figure 2 Expression and characterization of D-PCF molecule as a dengue vaccine candidate. (a) 4%–12% SDS–PAGE of purified D-PCF under reducing (R) and non-reducing (NR) conditions and detection by Coomassie staining (left panel), or immuno-detection by Western blotting and probing with anti-human IgG Fc, anti-dengue antigen and anti-CT specific antibodies, indicating presence of polymers (P), monomers (M) and single chains (S) (three right panels). (b) Size-exclusion chromatography (SEC) analysis of D-PCF in comparison with human monomeric IgG and pentameric IgM (c) Hydrodynamic diameter of D-PCF determined by dynamic light scattering using a Zeta-sizer; particle average Zeta-size (right) and the distribution by intensity (left) are shown.

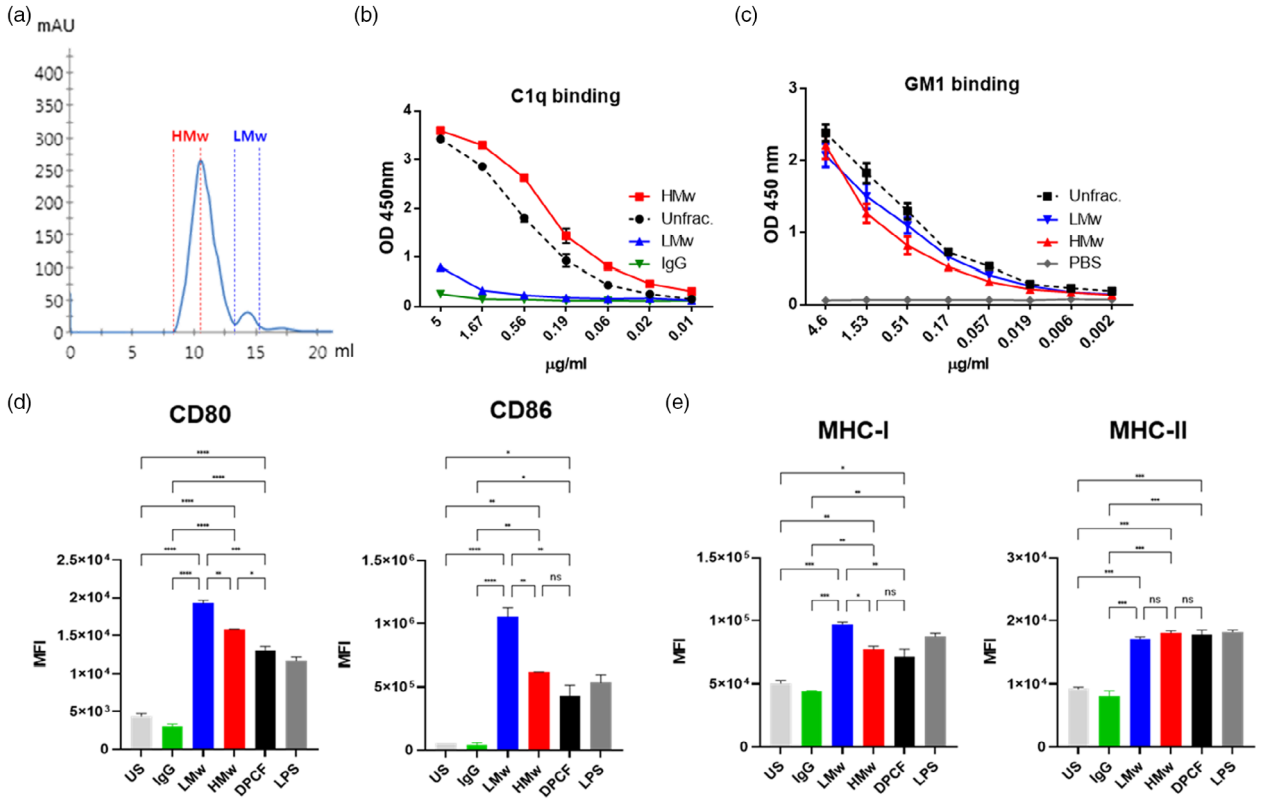

Figure 3 Fractionation and bio-physical properties of the assembled D-PCF. (a) SEC fractionation indicates presence of high-molecular weight (HMw) and low-molecular weight (LMw) proteins. (b) The C1q and (c) GM1 (e) binding capacity comparison between HMw, LMw and unfractionated (U) D-PCF. (d) Detection of co-stimulatory molecules and (e) MHC class-I and II after stimulation of monocyte-derived dendritic cells (moDCs) derived from human PBMC with 10 µg/mL of fractionated D-PCF (HMw and LMw); LPS was used as a positive and human monomeric IgG as a negative control. Shown are means of triplicate biological repeats + SD, with *indicating statistically significant difference (* < 0.05; ** < 0.01; *** < 0.001), by one-way Anova and Tukey's correction test.

fractionated by SEC (Figure 3a) and fractions were compared in three functional assays. In the first, we observed that while HMw and the unfractionated D-PCF (which is predominantly composed of HMw, as seen in Fig,3a) bound efficiently to C1q by ELISA, the LMw fraction bound to a much lesser degree (Figure 3b), indicating the superiority of HMw in activating the Complement. However, in the GM1 binding assay, there were no significant differences as both fractions (as well as unfractionated protein) displayed similar binding. We also tested these fractions for their potential to activate human monocyte-derived immature dendritic cells (DC), by measuring the expression of activation markers CD80 and CD86, as well as the MHC antigen presentation molecules. While both fractions were able to activate immature DC and increase the expression of the MHC molecules, somewhat surprisingly, the LMw fraction induced slightly higher expression of CD80, CD86 and MHC-I (HLA-ABC) than HMw, while expression of MHC-II (HLA-DR) was similar (Figure 3d,e). Considering that both fractions exhibited similar functional properties in these assays (apart from C1q binding) and that LMw constituted a low proportion in the D-PCF preparation, we used the unfractionated protein in our subsequent mouse immunization studies.

Binding to APC and FcγRIII receptor

As one of the key functions of Fcγ-fusion proteins as vaccine candidates is binding to APC via Fcγ receptors, we next investigated if our construct could bind to APC and be internalized. Using the human THP-1 monocyte/macrophage cell line as a proxy for APC, we could observe complete binding by flow cytometry, using two different concentrations of D-PCF (Figure 4a). While this assay did not specifically confirm that this binding is exclusively by the low- and high-affinity Fcγ receptors, we however, did demonstrate that D-PCF bound to immobilized low-affinity FcγRIII in a dose-dependent manner, by surface plasmon resonance assay (Figure 4b). This binding was far superior to that exhibited by human monomeric IgG, used as a comparator. To see if the binding of D-PCF leads to its internalization by APC, we employed confocal microscopy, where monomeric IgG again served as a comparator. As can be seen in Figure 4c, neither a fluorescently tagged secondary antibody alone nor IgG was internalized. In contrast, a very efficient internalization could be seen for D-PCF. Together, these data show that D-PCF can very effectively target APC via surface Fcγ receptors and that this leads to the uptake of the construct and subsequent antigen presentation.

Functional superiority of D-PCF over D-PIGS

Next, we wanted to compare the functional properties of D-PCF to an earlier iteration of polymeric fusion protein D-PIGS (Kim *et al.*, 2018), with the key difference being polymerization through CTB in D-PCF, and the IgM 'tail piece' in D-PIGS. This difference, however, also means that D-PCF was now capable of

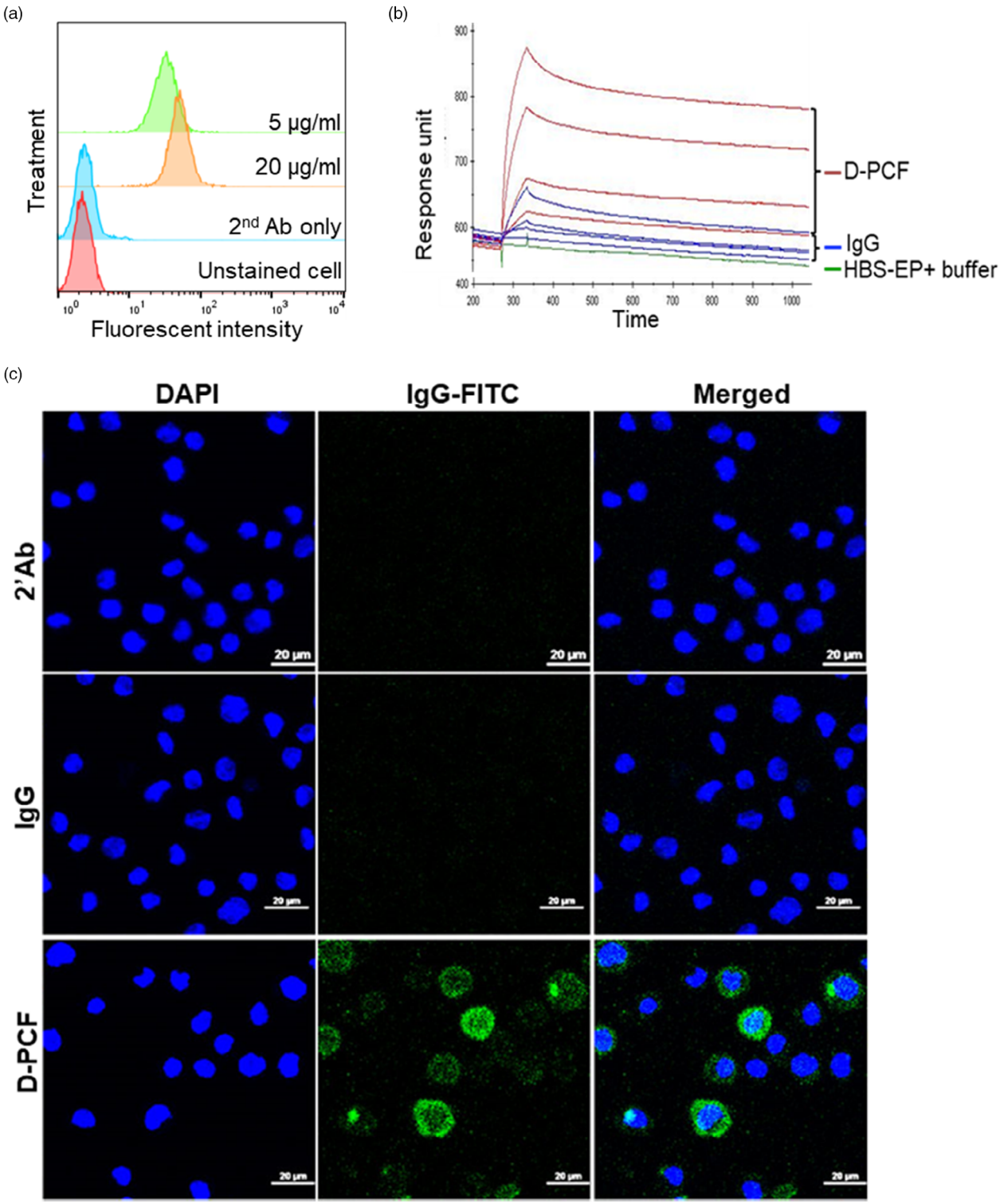

Figure 4 Functional characterization of D-PCF. (a) The APC-binding activity of D-PCF was demonstrated by binding to the surface of THP-1 cells at two doses (5 and 20 µg/mL). (b) Higher avidity of binding of D-PCF than monomeric IgG to a recombinant low affinity recombinant FcγRIII by surface plasmon resonance (with HBS-EP buffer as the background control). D-PCF and monomeric IgG were bound at 30, 10, 3.33 and 1.11 nM concentration. (c) Internalization of D-PCF by macrophage THP-1 cells by confocal microscopy; DAPI and anti-IgG-FITC were used to stain the nucleus (blue) and internalized antigen (green); monomeric IgG and anti-IgG-FITC (2'Ab) were used as the comparator and the negative staining control, respectively.

double targeting both epithelial surfaces (through CTB) and APC (through Fc). In a direct comparison using the same concentrations, we could observe that D-PCF was more efficiently internalized by THP-1 cells than D-PIGS (Figure 5a,b). D-PCF was also far more effective in activation of human moDC (as measured by CD80 and CD86 expression), and it induced higher

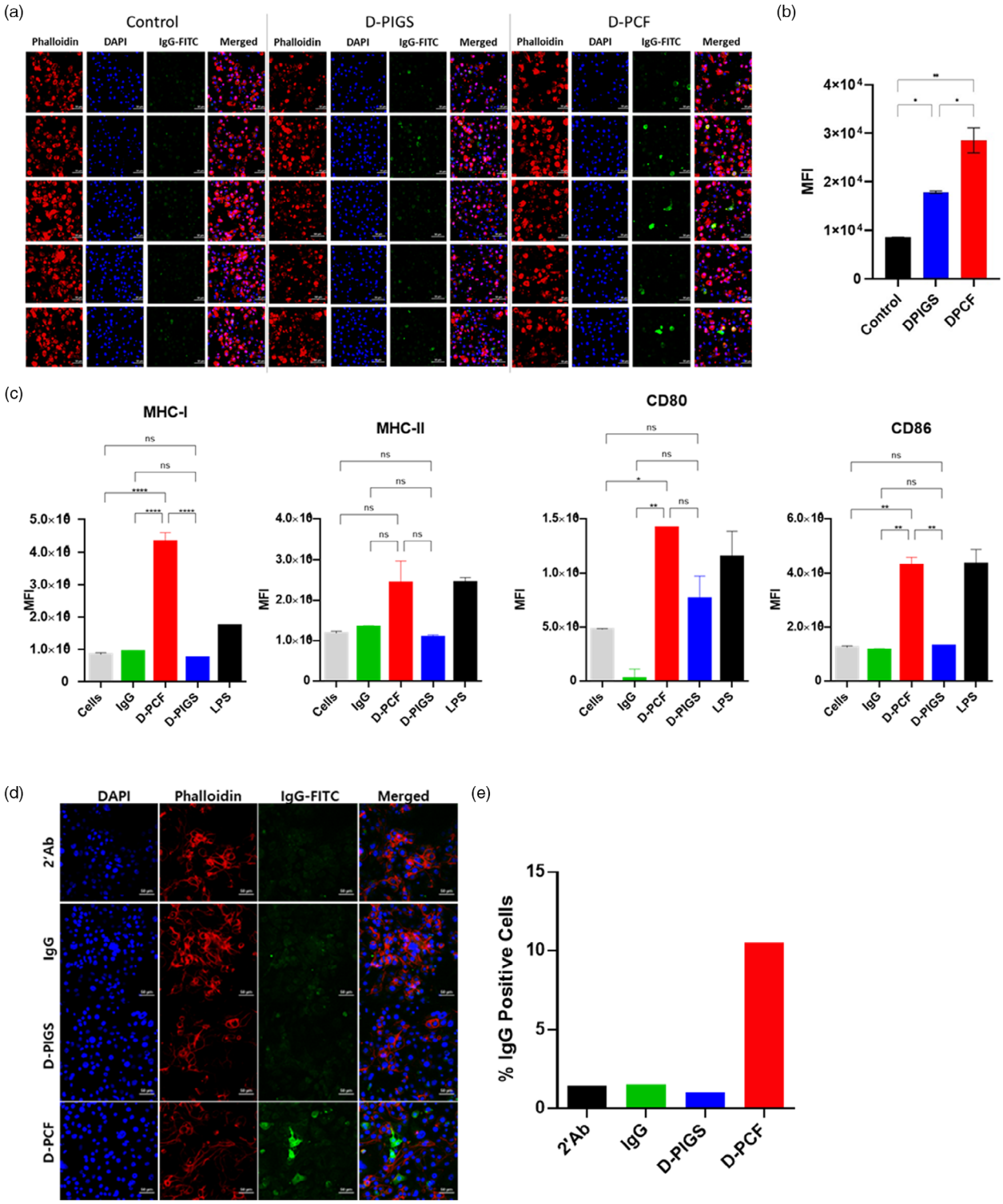

Figure 5 Enhanced binding/internalization of D-PCF over D-PIGS by APCs and human alveolar epithelial cells. (a)–(c) mo-DC & (d)–(e) hAEC; (a) and (c) show individual stainings and (b) and (e) summed up results from counting cells from multiple fields. (c) Comparison of D-PCF and D-PIGS for capacity to enhance MHC I and II expression and activate mo-DC. Comparators, controls and statistical analysis same as in Figure 3.

levels of MHC-I and II expression than D-PIGS (Figure 5c). Most strikingly though, while D-PIGS were completely inert when incubated with primary human alveolar epithelial cells, D-PCF could be seen internalized by these cells (Figure 5d,e), and we ascribe this property to the interaction of CTB with GM1 on these cells. Then, taken together, this shows that D-PCF was superior to D-PIGS in cellular interactions and therefore likely to carry a greater immunogenic potential *in vivo*.

Humoral and cellular immune responses in immunized mice

We ultimately tested our hypothesis of enhanced immunogenicity of D-PCF over D-PIGS by direct comparison in immunized mice (Figure 6). For this purpose, we used Balb/c mice transgenic for human high affinity Fcγ receptor (FcγRI, CD64), since D-PCF incorporates human IgG1-Fc portion. The immunization schedule is shown in Figure 6a and includes one subcutaneous injection and two intranasal boosts. No exogenous adjuvants were used as both vaccines were intended to be 'self-adjuvanting' by molecular design. In the first pilot study, only D-PCF was used to measure serum antibody responses after each immunization (Figure 6b), and it showed that they sharply increased after the first intranasal boost, and only marginally after the second boost. In the second mouse study, the two vaccine constructs were compared directly for the capacity to induce antibody and cellular responses. After each immunization, cEDIII-specific sera IgG responses were higher for D-PCF than for D-PIGS (Figure 6c). We also measured different antibody isotypes and subtypes in sera and BAL of immunized mice (Figure 6d,e). These analyses showed that D-PCF induced more IgG1, IgG2a and IgG2b in sera than D-PIGS, as well as total antigen-specific IgG and IgA (Figure 6d). The end-point titers for IgG subtypes and IgA are shown in the last panel of Figure 6d, showing that IgG1 was the predominant antibody species induced by both vaccines, reaching an end point titre of 100 000. Nevertheless, the endpoint titers were higher for D-PCF than D-PIGS for all antibody types and subtypes in sera, with IgG2a and IgA showing the greatest (>50-fold) difference. Similar trends were observed also for BAL antibodies (Figure 6e). Here, the most remarkable difference was observed for BAL IgA (and to a lesser extent IgG), with D-PCF inducing much higher responses than D-PIGS. Again, the last panel in Figure 6e shows that this superiority was reflected in at least 10-fold or higher endpoint titers for all antibody isotypes and subtypes.

We next analysed cellular immune responses in the spleens of immunized mice (Figure 6f). Since we observed the presence of both Th1 (IgG1) and Th2 (IgG2a) IgG antibody subtypes in sera and BAL (Figure 6d,e), we wished to see whether this would also be reflected in cytokine profiles of splenic T cells culture supernatants in the antigen recall assays. As shown in Figure 6f, no significant differences between D-PCF and D-PIGS were observed for IL-2 (Th1) and IL-10 (Treg), but significantly higher responses for D-PCF were measured for IFN-γ (Th1), IL-4 (Th2) and IL-17 (Th17). In summary, these mouse studies showed conclusively that D-PCF is considerably more immunogenic than D-PIGS, and that it induced both systemic and mucosal antibody responses, as well as splenic T cells responses of mixed Th1/Th2/Th17 phenotype.

Antibody cross-reactivity against 4 serotypes of dengue EDIII

Since in our study we used the consensus EDIII (cEDIII) sequence representing all four serotypes of dengue, we wanted to see whether the induced antibodies could recognize each individual serotype. Mouse sera and BAL from D-PCF or D-PIGS immunized

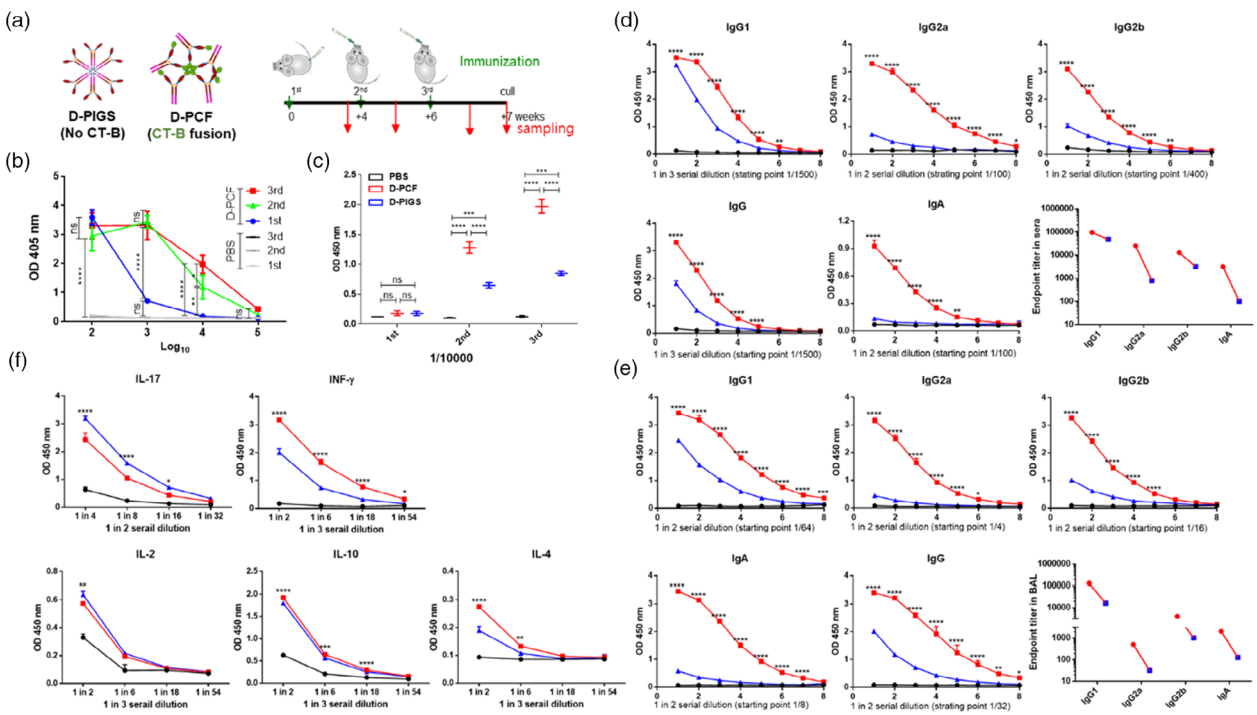

Figure 6 Humoral and cellular responses induced in mice after immunization with either D-PCF or D-PIGS. (a) Schematic depiction of immunization schedule in transgenic CD64 mice (n = 3) (b) The kinetics of serum IgG response after each immunization with D-PCF, with the X-axis showing 10-fold serial dilutions and Y-axis relative optical density. (c) Comparative kinetics of serum IgG responses induced by D-PIGS and D-PCF shown for 1:10 000 dilution point. (d) and (e) Antibody isotype and sub-type responses in pooled sera (d) and BAL fluids (e) from D-PCF (red), D-PIGS (blue) and mock-immunized mice (black), with serial dilutions indicated, and the last panels showing the end-point titers. (f) Cytokine responses in splenocytes cultures from immunized mice after stimulation with cEDIII antigen. Shown are the means and SD from triplicate measurements. Statistical analysis performed by Two-way ANOVA and Tukey's multiple comparison correction test (* < 0.05; ** < 0.01; *** < 0.001; **** < 0.0001).

mice were therefore tested for reactivity with each serotype-derived EDIII by ELISA (Figure 7). Sera from mice immunized with either construct showed reactivity with all four serotypes (Figure 7a) but apart from serotype 4, this was significantly higher for D-PCF than D-PIGS, with serotype 1 showing the greatest difference. This was then also reflected in endpoint titers for each serotype (Figure 7c), with D-PCF induced antibodies showing consistently higher values than those induced by D-PIGS.

Aerosolization of D-PCF

Finally, since we observed a remarkable benefit of D-PCF in terms of mucosal (intranasal) vaccine delivery and induced mucosal and systemic responses, we investigated if the construct could be aerosolized without loss of protein and activity, as a pre-requisite for mucosal vaccine delivery, by inhalation. Using a commercial Omron MicroAIR U22 nebulizer designed for oral respiratory delivery, we demonstrated that the recovery of protein in the condensate was 93% (Figure 8a). We then tested the protein before (input) and after (output) aerosolization in three functional assays, namely, APC (Figure 8b), C1q (Figure 8c) and GM1 (Figure 8d) binding. In each case, it could be seen that there were no significant differences in protein binding, before and after aerosolization. This demonstrates the feasibility of the D-PCF construct as a mucosal as well as systemic vaccine candidate.

Discussion

In this study, we report on a novel plant-expressed vaccine delivery platform designed for inducing robust systemic and mucosal immunity and show that it is very highly immunogenic in mice without additional adjuvants. The induced responses were vastly superior to those observed with a previous iteration of an Ig-Fc-fusion protein-based vaccine, in a direct head-to-head comparison in mice.

Our approach to designing this vaccination concept was empirical, taking advantage of the simplicity of a single polypeptide-based vaccine formulation, and the functional complexity of its interaction with different cell types at the site of delivery, especially the mucosa. Thus, the first important consideration was for the vaccine to engage the mucosal epithelial cells by inclusion of CTB, a potent molecular immune modulator, but without the prohibitive inflammatory response of the holotoxin, or the risk of binding to olfactory sensory neurons, located in the nasal epithelium. Indeed, the latter is the main concern for type AB_5 enterotoxins in mucosal vaccines for humans, as they can potentially induce adverse effects as observed in a clinical trial of a nasal HIV vaccine formulated with the heat-labile *E. coli* toxin, LT-I (Adjuvant LTK63) (Lewis *et al.*, 2009). However, removal of the A subunit (enzyme ADP-ribosyl transferase) renders cholera toxin much safer while retaining the immunomodulatory activity. Furthermore, our preferred aerosolized route of vaccine delivery is through the mouth rather than the nose, circumventing further any risk of binding to olfactory neurons. Indeed, our preliminary *in vitro* modelling studies using a mash nebulizer (rather than nasal spray) demonstrated excellent feasibility of this mode of aerosolized delivery through a replaceable mouthpiece attached to the nebulizer, with more than 95% of the protein and its activity

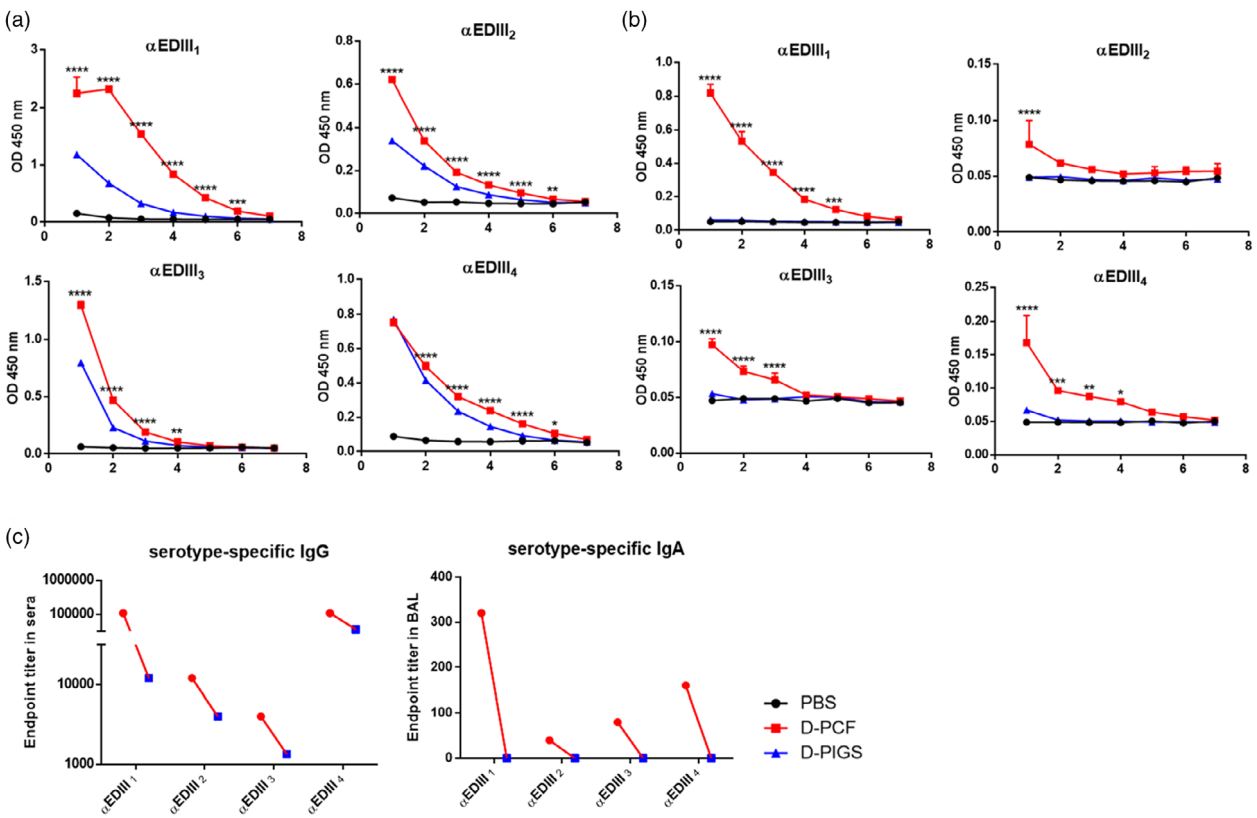

Figure 7 Antibody cross-reactivity against 4 different serotypes of EDIII. Serum IgG (a) and BAL IgA (b) antibodies reactivity to each dengue serotype were compared between D-PIGS and D-PCF (****P value = 0.0001). (c) The comparison of the end-point titers for serum IgG and BAL IgA is shown. (a) and (b) The experimental groups immunized with D-PCF, D-PIGS or PBS are shown in red, blue and black, respectively. Statistical analysis as in Figure 6.

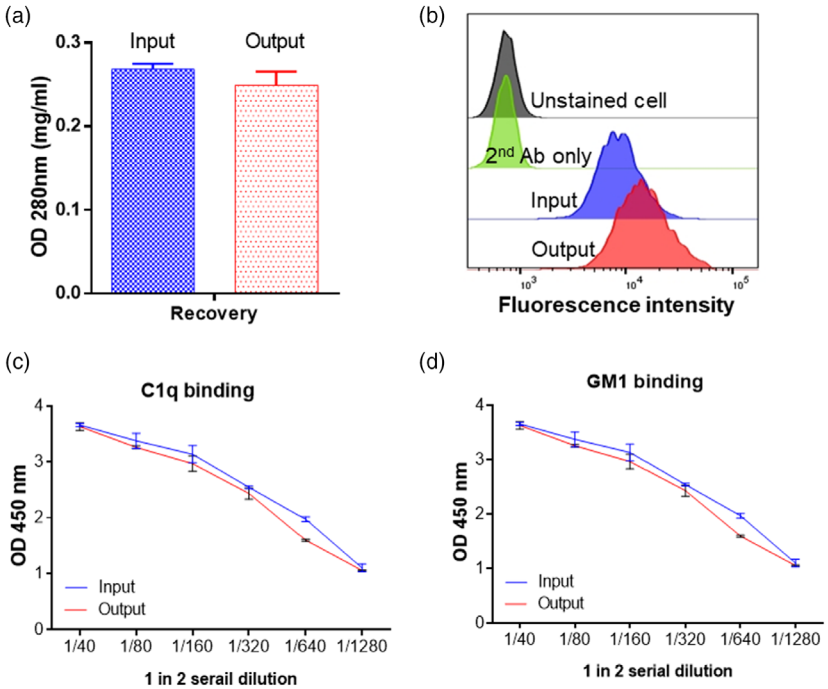

Figure 8 Aerosolization of D-PCF and analyses of bio-functionality after nebulizing. (a) D-PCF formulated in PBS with 0.01% polysorbitol-80 as an excipient was nebulized in an OMRON MicroAIR device (U22, Japan). Blue and red colours indicate D-PCF properties before and after nebulization. (b) Protein recovery from the condensated aerosol was measured by nanodrop at OD 280 nm. (c–e) Comparative binding to C1q (c), GM1 (d) and APCs (e) before and after nebulization is shown, respectively. Means and SD from triplicate measurements are shown.

recovered in the condensate. However, the construct could be used also as an injectable vaccine, as CTB has been shown previously to induce robust systemic immunity when co-administered with antigens (Antonio-Herrera *et al.*, 2018), or indeed by a combination of systemic prime and mucosal boost, as demonstrated in this study.

The second consideration is the inclusion of the polymeric Ig-Fc molecules for efficient targeting of APC through Fcγ receptors on their surfaces. It has been known for a long time that immune complexes (ICs) are potent immunogens and that during an infection they amplify the immune response to the pathogen (Heyman, 1990). Ics serve as a bridge between innate and adaptive immune cells, by engaging Fc receptors on APC such as dendritic cells and macrophages, which facilitates the uptake, processing and presentation of antigens via the MHC-II presentation pathway to helper CD4 T cells, which in turn support development of CD8 T cells and B cells. However, natural IC are not feasible to isolate or make in a reproducible manner *in vitro*, as that would require polyclonal sera or a cocktail of monoclonal antibodies. To circumvent these issues, many different approaches have been attempted, resulting in varying degrees of success (recently reviewed in (Tang *et al.*, 2021)). We ourselves employed molecular engineering techniques and first pioneered the concept of recombinant IC (RIC) based on a single monoclonal antibody expressed in plants, (Chargelegue *et al.*, 2005; Pepponi *et al.*, 2014), an approach that was also adapted by other research groups (Phoolcharoen *et al.*, 2011). However, the RICs approach suffered from being non-universal as it required antigen-specific antibody cloning for each different antigen. The next iteration of IC-like Fc-based fusion proteins was the 'PIGS' (Poly-Immuno-Globulin-Scaffold), which dispense with the antibody-binding domains altogether and instead, multimerization is achieved by insertion of the IgM tail piece to the C-terminus of the Fc-γ polypeptide, resulting in hexamerization of the construct much as IgM does (Kim *et al.*, 2015, 2018; Webster *et al.*, 2018). While immunogenic systemically, the PIGS constructs however failed to induce strong mucosal immune responses (Webster *et al.*, 2018), when delivered either systemically or mucosally, and we speculated that this was due to their restricted access to APC in the absence of an adjuvant. Therefore, the present construct (D-PCF) addresses that issue by inclusion of CTB within the same polypeptide, which now displays dual functionality, i.e., recruitment of APC by CTB and APC binding and uptake via the Fc.

The third consideration in our vaccine design was the structural demands of this relatively complex 3-component molecule, so as not to hinder the function of any individual component, or indeed the polymerization of the whole complex. For this, the linking sequences between each component are important. We used hexamer sequence Gly-Pro-Gly-Pro-Gly-Ser to link CTB and the antigen, partly based on a report by Chen et al. who investigated the preference of amino acid residues in natural linkers by calculating the ratio of single amino acid occurrence within the linker (Chen *et al.*, 2013), and threonine, serine, proline, glycine, aspartic acid, lysine, glutamine, asparagine and alanine were suggested to be preferable. On the other hand, for linking the antigen with the Fc, in addition to retaining the Fc-associated glycan site which controls structural flexibility (Ackerman *et al.*, 2016; Kajihara *et al.*, 2012) we extended the Fc structure to achieve the minimal Ig-like 'Y' shape without a light chain, by the inclusion of last β-stand c-terminus of the CH1 domain, and additionally the heavy chain 'hinge' region sequence. Thus, this minimal Ig-Fc structure allows not only dimerization of the two heavy chains but is also sufficient for binding to all type I FcRs, as

well as the C1q component of the complement, (defined by the sequence $_{233}$PAPELLGGP$_{241}$ in IgG1) (Sondermann *et al.*, 2000; Ugurlar *et al.*, 2018; Vidarsson *et al.*, 2014) which initiates the classical complement pathway. We demonstrated that D-PCF bound to both FcγRs and C1q very efficiently, which means that the construct could be captured by APC either directly via FcγRs on their surface or by the complement receptors (CR1 and CR2) following complement activation. For this reason, we used the human IgG1 subtype, as it has the highest affinity for C1q and FcgRs (Sondermann *et al.*, 2000; Ugurlar *et al.*, 2018).

Apart from these functional and structural considerations, we also considered issues of importance for D-PCF expression and assembly in plants specifically. PCF platform carrying three hetero components requires optimal efficiency of protein folding and minimized exposure to endogenous plant cell proteases to achieve high-level expression and assembly. The D-PCF molecule has two putative N-glycosylation sites on both CTB (Matoba, 2015) and IgG-Fc portion (Higgins, 2010), thus ER-targeting may also increase the glyco-homogeneity of the expressed construct. Thus, the expression in ER was preferred as there are abundant chaperones such as Hsp70-type chaperone and BiP (binding-immunoglobulin protein aka GRP-78), while less protease activity is present in ER compared to different subcellular locations (van der Hoorn, 2008).

ER retention strategy was used also to devoid the protein of plant-specific, potentially immunogenic β1,2-xylose and core α1,3-fucose modifications. Glycosylation has a critical role in controlling the cellular activity of an antibody, especially the FcR-binding function (Jennewein and Alter, 2017). For example, the absence of fucose on IgG1 allows it to bind with 10–100-fold higher affinity to human FcγRIIIa and FcγRIIIb, resulting in greater activation of cells (Shields *et al.*, 2002; Subedi and Barb, 2016). The ER-retained proteins in plants contain only the high mannose type N-glycans (Man-9) without added further sugars, which makes them structurally and functionally more like mammalian cells glycoprotein precursors (Ko *et al.*, 2003), thus more amenable to human application than plant-glycosylated proteins.

With these considerations in mind, we were able to express fully assembled D-PCF molecules in plants and purify them to a high degree of homogeneity by a simple protein-A chromatography step, owing to the presence of IgG-Fc within the construct. Surprisingly, subsequent size-exclusion chromatography analyses revealed that most of the protein was in high-molecular weight (polymeric) form, with lower molecular weight species constituting less than 10% of the total protein. This was a significant improvement compared to D-PIGS, which rely on polymerization through the IgM 'tail piece', and which yielded less than 50% of assembled polymers (Kim *et al.*, 2018), suggesting that polymerization of the Fc-fusion proteins through CTB is a more robust process. Considering that D-PCF in purified form consisted of predominantly HMW polymers, we, therefore, performed subsequent *in vitro* and *in vivo* experiments with the unfractionated protein. We could demonstrate efficient APC binding and complement activation, but unlike D-PIGS, D-PCF could also bind to gangliosides, a gain of function that is critical for the improved immunogenicity of the present construct, as we demonstrated in mouse immunization experiments.

Thus, in our mouse studies, we demonstrated the marked superiority of D-PCF over D-PIGS for inducing both systemic and mucosal immunity, when combining the systemic prime and intranasal boost regimen. Considering that a large proportion of the human population in dengue-endemic areas has already been exposed to dengue, or may have been vaccinated, we think that a mucosal boosting with a vaccine like D-PCF could bring the overall level of immunity further up, to a level sufficient to prevent future infections. Or alternatively, systemic priming of naïve hosts (young children included) followed by mucosal boosting may prove to be an equally efficacious strategy. Based on our mouse studies, exceptionally high (i.e., 10^5) IgG end-point titers in both sera and BAL could be achieved by the prime-boost regimen, although surprisingly the IgA levels in BAL were significantly lower. This regimen also induced strong cellular responses as demonstrated by secretion of both Th1 and Th2 cytokines by splenocytes of immunized mice, upon antigen recall assays.

A limitation of our study may be that while using the serotype consensus sequence of the envelope glycoprotein domain III in our construct simplified the vaccine design, this approach may not be equally protective against all four dengue virus serotypes, and a tetravalent vaccine based on the four individual serotypes may be more protective. This is something that needs to be determined in future studies, as well as if the vaccine may pose any risk of antibody-dependent enhancement of infection (ADE). However, as a proof of concept, we demonstrated that robust immunity against dengue can be induced using this novel vaccine approach. The protein-only nature of our vaccine lends itself well to GMP production and safe adjuvant-free human application. While dengue is not a respiratory disease there is evidence of a significant mucosal involvement during pathogenesis of the infection (Thomas *et al.*, 2010) and also of intestinal injury in patients with severe dengue infection (Vejchapipat *et al.*, 2006). Vaccine boosting of systemic immunity while also providing additional mucosal immune component would therefore be highly desirable. The intended aerosolized delivery by inhalation would be particularly amenable for seasonal, nationwide vaccination campaigns in a relatively short space of time, due to ease of administration and reduced need for needle-trained clinical personnel.

In summary, D-PCF is a new and highly immunogenic protein-only based vaccine platform that derives its own adjuvanticity autonomously, making it a safe vaccine candidate for human testing and application. We showed that it has superior immunogenicity over other Fc-fusion proteins in mice in a systemic-prime, mucosal-boost vaccination regimen and we demonstrated the feasibility of its aerosolized delivery. We believe that this novel vaccination concept merits further development and testing, including in clinical trials in humans.

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Authors contribution

MYK conceived the concept, performed the cloning and majority of experimental analyses of D-PCF and co-wrote the manuscript; EJV and ACT performed confocal microscopy and flow cytometry studies; MJP performed SPR analyses; THK provided input on structural studies; JKM assisted with plant expression studies; YSJ conceived the project with MYK and RR and contributed to

analyses and writing of the manuscript; RR conceived the project, performed mouse immunizations and co-wrote the manuscript with MYK and YSJ.

Conflict of interest

THK is the CEO of GeneCell Biotech company, in Jeonju, Korea; all other authors declare no conflict of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Supporting Information.