

Secretion of dengue virus envelope protein ectodomain from mammalian cells is dependent on domain II serotype and affects the immune response upon DNA vaccination

J. L. Slon Campos, M. Poggianella, S. Marchese, M. Bestagno and O. R. Burrone

International Centre for Genetic Engineering and Biotechnology, Trieste, Italy

Correspondence

O. R. Burrone
burrone@icgeb.org

Received 21 July 2015
Accepted 4 September 2015

Dengue virus (DENV) is currently among the most important human pathogens and affects millions of people throughout the tropical and subtropical regions of the world. Although it has been a World Health Organization priority for several years, there is still no efficient vaccine available to prevent infection. The envelope glycoprotein (E), exposed on the surface on infective viral particles, is the main target of neutralizing antibodies. For this reason it has been used as the antigen of choice for vaccine development efforts. Here we show a detailed analysis of factors involved in the expression, secretion and folding of E ectodomain from all four DENV serotypes in mammalian cells, and how this affects their ability to induce neutralizing antibody responses in DNA-vaccinated mice. Proper folding of E domain II (DII) is essential for efficient E ectodomain secretion, with DIII playing a significant role in stabilizing soluble dimers. We also show that the level of protein secreted from transfected cells determines the strength and efficiency of antibody responses in the context of DNA vaccination and should be considered a pivotal feature for the development of E-based DNA vaccines against DENV.

INTRODUCTION

Dengue disease is a mosquito-borne viral infection caused by dengue virus (DENV), one of the most important human pathogens affecting millions throughout the tropical and subtropical regions of the world (Murray *et al.*, 2013; WHO, 2009). DENV infection produces a systemic disease with manifestations that range from non-symptomatic or mild flu-like syndrome (dengue fever) to severe and potentially fatal haemorrhagic manifestations (dengue haemorrhagic fever and dengue shock syndrome) (Gulland, 2013; Naish *et al.*, 2014; WHO, 2009).

DENV belongs to the family *Flaviviridae* and is composed of four closely related serotypes: DENV1, DENV2, DENV3 and DENV4 (Bäck & Lundkvist, 2013; Weaver & Vasilakis, 2009). Like all flaviviruses, DENV is an enveloped virus with a positive sense ssRNA genome of approximately 11 kb that encodes ten viral proteins: seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) and three structural ones (C, Pr-M and E) (Ninth Report of the International Committee on Taxonomy of Viruses, 2012; Simmons *et al.*, 2012). Of these, the envelope glycoprotein (E), a class II viral membrane fusion protein, is the

major constituent of the viral particle (Ge & Zhou, 2014; Modis, 2014; Schibli & Weissenhorn, 2004). E is approximately 500 aa in length and fulfils essential functions regarding host range, tropism, virus–cell attachment, cellular entry and viral assembly (Butrapet *et al.*, 2011; Lindenbach *et al.*, 2007). E folds into an elongated rod-like structure forming antiparallel homo-dimers, organized in a herringbone-like configuration on the viral surface (Mukhopadhyay *et al.*, 2005; Zhang *et al.*, 2003).

The E ectodomain, also termed soluble E (sE), involves approximately the first 400 aa and comprises three different structural domains named domain I (DI), DII and DIII (Rey *et al.*, 1995; Rouvinski *et al.*, 2015; Zhang *et al.*, 2004). While DIII is encoded in a single genomic sequence within the viral genome, DI and DII (DI/DII) are discontinuous with respect to the protein and encoded in intercalated genomic segments (three for DI, two for DII) (Modis, 2014). DI folds into a β -barrel structure with an axis parallel to the viral membrane and occupies a central position in the mature monomer (Modis *et al.*, 2005; Rey *et al.*, 1995). DII forms an elongated finger-like structure with a stable core that expands distally in two loops (Rey *et al.*, 1995; Zhang *et al.*, 2004); the most distal one carries a hydrophobic glycine-rich sequence that serves as the internal fusion loop during fusion to host cell membranes

Four supplementary figures are available with the online Supplementary Material.

(Allison *et al.*, 2001). Additionally, DII provides the surface where the main interactions for E dimerization occur (Modis *et al.*, 2005; Rey *et al.*, 1995). The C-terminal DIII domain has an Ig-like β -barrel structure with a hydrophobic inner surface in a pocket that accommodates the fusion loop of the opposing monomer (Allison *et al.*, 2001; Erb *et al.*, 2010). DIII is also believed to contain the receptor-binding sites to the host cell (Erb *et al.*, 2010; Mukhopadhyay *et al.*, 2005) and has been implicated in determining host range, tropism and virulence (Lindenschmidt *et al.*, 2007). A hinge region formed by the four strands that span between the different DI and DII coding segments provides the flexibility for E conformational changes during virus maturation (Butrapet *et al.*, 2011; Monath *et al.*, 2002), while a linker of 11 aa connects DI to DIII and is fundamental for proper E folding (de Wispelaere & Yang, 2012).

E protein is the main target of the antibody immune response during infection (Rothman, 2011; Wahala & Silva, 2011). While neutralizing epitopes have been found on all three domains and on the dimer interface (Dejnirattisai *et al.*, 2015; Rothman, 2011; Rouvinski *et al.*, 2015; Sukupolvi-Petty *et al.*, 2010), antibodies against the upper lateral surface of DIII are described as the ones with the highest neutralizing capacity (Crill & Roehrig, 2001; Gromowski *et al.*, 2008; Lok *et al.*, 2008; Matsui *et al.*, 2009). We have recently shown that DNA vaccination with DIII-based constructs of all four serotypes results in highly serotype-specific neutralizing responses without induction of antibody-dependent enhancement of infection (Poggianella *et al.*, 2015).

Genetic vaccination is a simple and efficient technique with high safety standards, low costs of production, excellent stability and capable of inducing robust antibody responses (Khan, 2013; Shedlock & Weiner, 2000; Vaughn *et al.*, 2008). In this approach, delivery of plasmid DNA encoding the antigen of choice results in antigen expression by cells of the host (Khan, 2013).

Here we present a detailed biochemical analysis of expression, secretion and folding of sE from all four DENV serotypes in mammalian cells, and how this affects their ability to induce antibody responses in DNA vaccinated mice. We show that DII plays a pivotal role in sE secretion, while DIII is responsible for stabilizing sE dimers. We also show that the sE secretion levels determine the strength of the antibody response, an important feature for the development of E-based DNA vaccines.

RESULTS

Secretion of E ectodomain depends on DII

Secretion of DENV recombinant sE from mammalian cells was investigated by overexpressing DNA constructs encoding the first 416 (for DENV1, DENV2 and DENV4) or 414 (for DENV3) amino acids of E proteins from the four different

serotypes with a signal leader peptide at the N terminus. The SV5 tag was included at the C terminus for detection. Schematics of the constructs used are shown in Fig. 1(a) and the corresponding sE amino acid sequences in Fig. S1 (available in the online Supplementary Material). sE secretion was analysed by Western blotting (WB) of cellular extracts and supernatants of transfected HEK293T cells. Clear differences in the secretory phenotypes of the distinct sE serotypes were observed (Fig. 1b). While sE from serotypes 1 (1sE) and 2 (2sE) were poorly secreted and accumulated in the cellular extracts (see Fig. 1b long-exposure), sE from serotypes 3 (3sE) and 4 (4sE) were better secreted. These secretory phenotypes were independent of the mammalian cell line used (Fig. S2).

To investigate whether the poor-secretion phenotype of 1sE and 2sE was dependent on DI/DII or DIII, we independently tested constructs encoding these two parts for all serotypes. Fig. 1(c) shows that DI/DII of serotypes 1 and 2 (1DI/DII and 2DI/DII) were not efficiently secreted, resembling phenotypes of the corresponding sE proteins. In contrast, DI/DII of serotypes 3 and 4 (3DI/DII and 4DI/DII) were both secreted, as their homologous sE. On the other hand, DIII was secreted, albeit in low amounts, in serotypes 1 and 3. Thus, secretion of sE appeared to be determined by DI/DII.

This was confirmed with sE chimeras that contained DI/DII and DIII from different serotypes. Four chimeric constructs were obtained: (i) DI/DII of the poor-secretory serotypes (serotype 1 and serotype 2) and DIII from serotype 3 [1sE(3DIII) and 2sE(3DIII), respectively]; (ii) DI/DII from serotype 3 and DIII from serotype 2 [3sE(2DIII)]; and (iii) DI/DII from serotype 4 and DIII from serotype 3 [4sE(3DIII)]. As shown in Fig. 1(d), the two chimeric sE containing DI/DII from the poor-secretory serotypes 1 and 2 were not efficiently secreted. In contrast, DI/DII from serotype 3 allowed secretion of the 2DIII-containing sE chimera. The 4sE(3DIII) chimera served as a secretory chimeric control that was secreted, as expected. Thus, DI/DII determines sE secretion.

To further map the secretory phenotype, we tested DI/DII chimeras where the hinge regions between the two domains belonged to the same serotype as DI. The 2DI/3DII chimera was well secreted, while 3DI/2DII was not, suggesting that the poor-secretory phenotype was exclusively dependent on DII (Fig. 2a). This was confirmed by analysing DI and DII sE chimeras. Grafting DI from serotype 3 into a 2sE [2sE(3DI)] did not improve secretion, while grafting of 3DII [2sE(3DII)] did (Fig. 2b, lanes 3–6). Conversely, grafting DI from serotype 2 into a 3sE [3sE(2DI)] did not affect secretion, while grafting of 2DII [3sE(2DII)] reduced it to near-undetectable levels. In these chimeras, the hinge regions between DI and DII belonged to the DI serotype (Fig. 2b, lanes 9–12). The sE secretory phenotype was therefore dependent on DII.

We then probed the folding state of the different proteins using mAb 4G2, which recognizes a DII conformational epitope on the fusion loop of all serotypes (Henchal

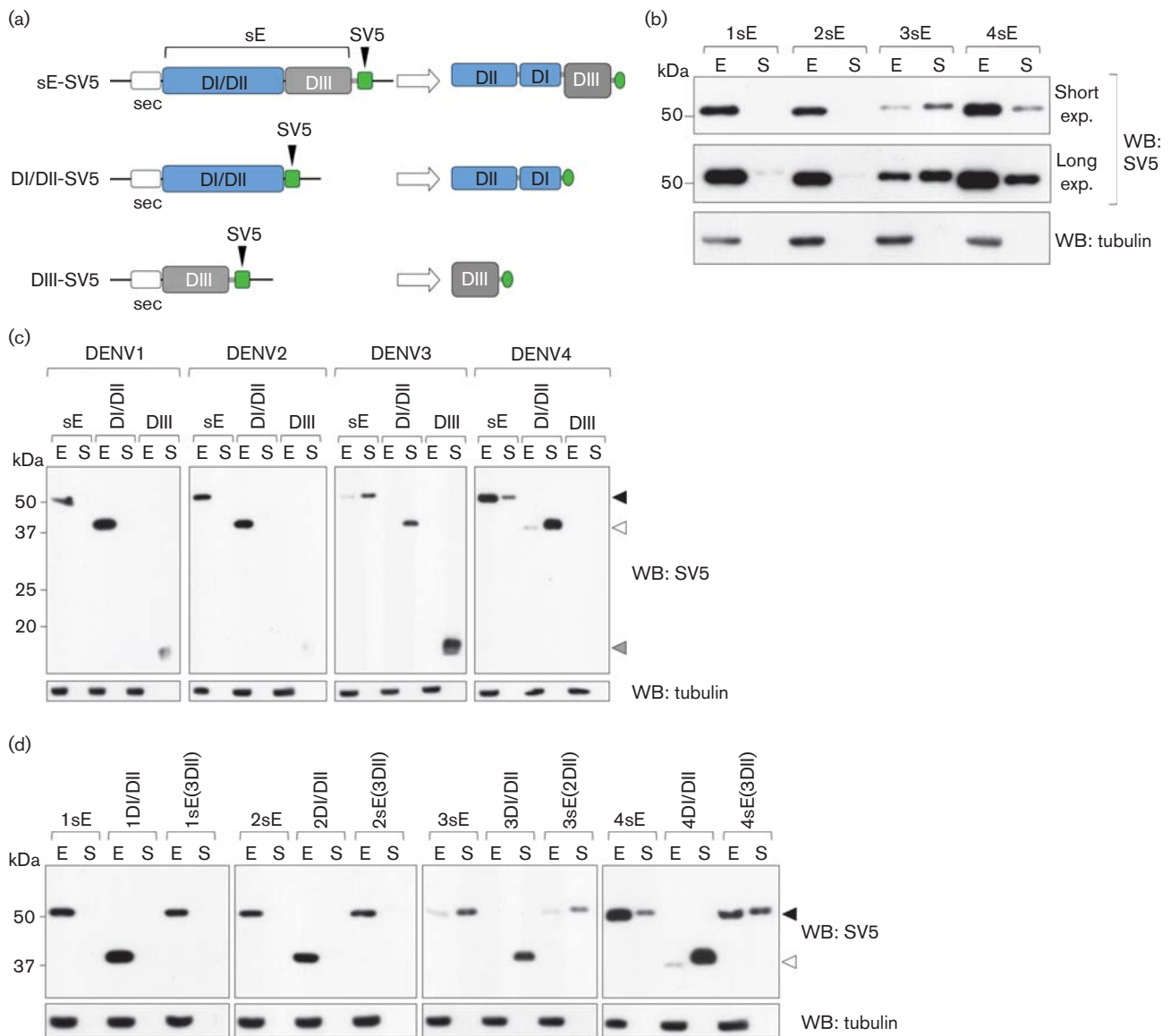


Fig. 1. Serotype dependent sE secretory phenotypes from mammalian cells. (a) Schematic of constructs and their products; sec corresponds to a signal leader peptide and SV5 (green) to the C-terminal tag. DI/DII are indicated in blue and DIII in grey. (b) WB of cellular extracts (E) and supernatants (S) of HEK293T cells transfected with sE constructs of the indicated serotypes. Short and long exposures are indicated. (c) WB of cells transfected with sE, DI/DII and DIII constructs of the indicated serotypes. (d) WB of cells transfected with the indicated DI/DII-DIII chimeras. For comparison, the corresponding sE and DI/DII proteins were analysed in parallel. Anti-tubulin was used as a loading control. Filled, open and grey arrowheads indicate sE, DI/DII and DIII proteins, respectively.

et al., 1985; Lin *et al.*, 2012). 4G2 recognized secreted DI/DII and sE proteins from DENV3 and DENV4, as well as the secreted chimeras 2DI/3DII and 2sE(3DII) (Fig. 3a). The same proteins were not recognized upon denaturation, confirming the conformational nature of the epitope. In contrast, DI/DII and sE from the poor-secretory DENV1 and DENV2, and chimeras 3DI/2DII and 3sE(2DII), were mostly not recognized by 4G2, in contrast

to positive controls 3DI/DII and 3sE (Fig. 3b). These results indicate a correlation of the secretory phenotype with the folding state of the protein. This was further confirmed with the non-glycosylated mutants (N67Q and N153Q) of serotype 3 DI/DII and sE that, in contrast to the glycosylated proteins, showed impaired secretion (Fig. 3c). As expected, the two poor-secretory proteins were not recognized by 4G2 (Fig. 3d).

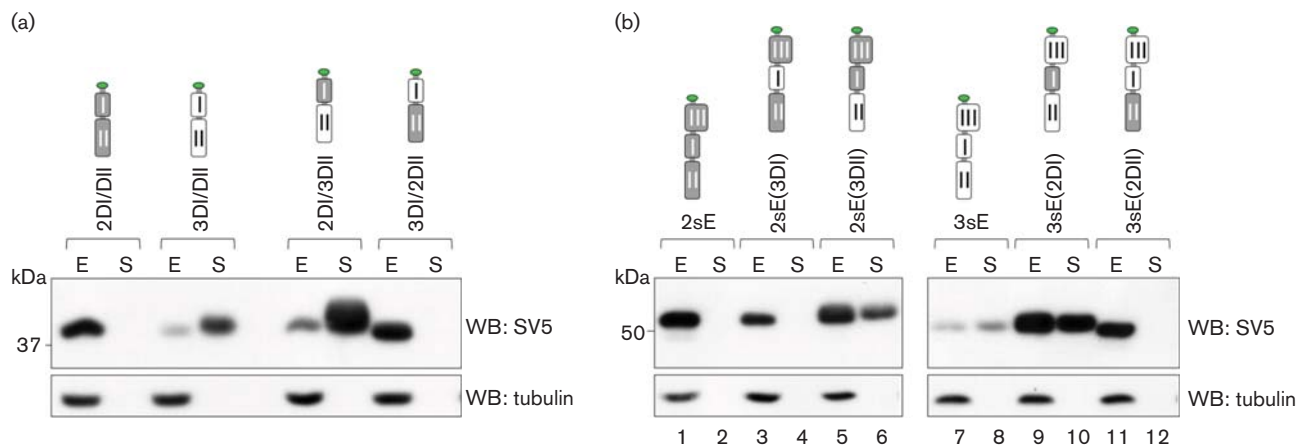


Fig. 2. Secretory phenotypes of sE and DI/DII proteins are DII-dependent. WB of cellular extracts (E) and supernatants (S) of HEK293T cells transfected with (a) DI/DII chimeras (2DI/3DII and 3DI/2DII), and (b) sE chimeras [2sE(3DI), 2sE(3DII), 3sE(2DI) and 3sE(2DII)]. In both cases, WT 2sE and 3sE are shown as controls; anti-tubulin was used as a loading control.

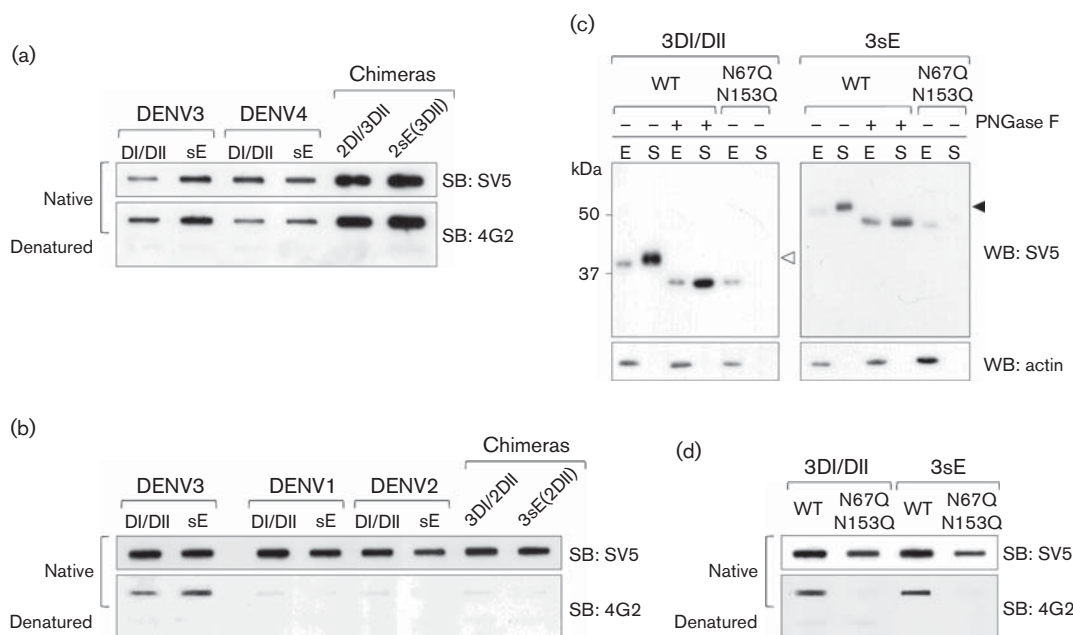


Fig. 3. DII folding is compromised in poor-secretory sE and DI/DII proteins. Slot blot of supernatants (a) of HEK293T cells transfected with DI/DII and sE constructs of serotypes 3 and 4 and chimeras 2DI/3DII and 2sE(3DII), and of cellular extracts (b) of cells transfected with DI/DII and sE constructs of serotypes 1 and 2 (and serotype 3 as control) and chimeras 3DI/2DII and 3sE(2DII). Upper panels correspond to samples reacted with anti-SV5 and lower panels to samples in native and denatured conditions (as indicated) reacted with mAb 4G2. (c) WB of cellular extracts (E) and supernatants (S) of cells transfected with the non-glycosylated 3DI/DII and 3SE mutants (N67Q; N153Q) and the WT controls. Samples treated with PNGaseF are indicated. Filled and open arrowheads indicate sE and DI/DII proteins, respectively. (d) Slot blot of cellular extracts containing WT 3DI/DII and 3sE and the non-glycosylated mutants, probed with anti-SV5 or mAb 4G2, on native or denatured samples, as indicated.

Interestingly, the DI/DII secretory phenotype was only temperature-dependent for serotype 2. Transfected cells were maintained at 37 °C for 16 h and shifted to 28 °C for the next 24 h. Total cell extracts and supernatants

were then analysed by WB (Fig. S3a). 2DI/DII was secreted at 28 °C but not at 37 °C, while secretion of the control 3DI/DII was not affected (Fig. S3b). As expected, 2DI/DII produced at 28 °C, but not the

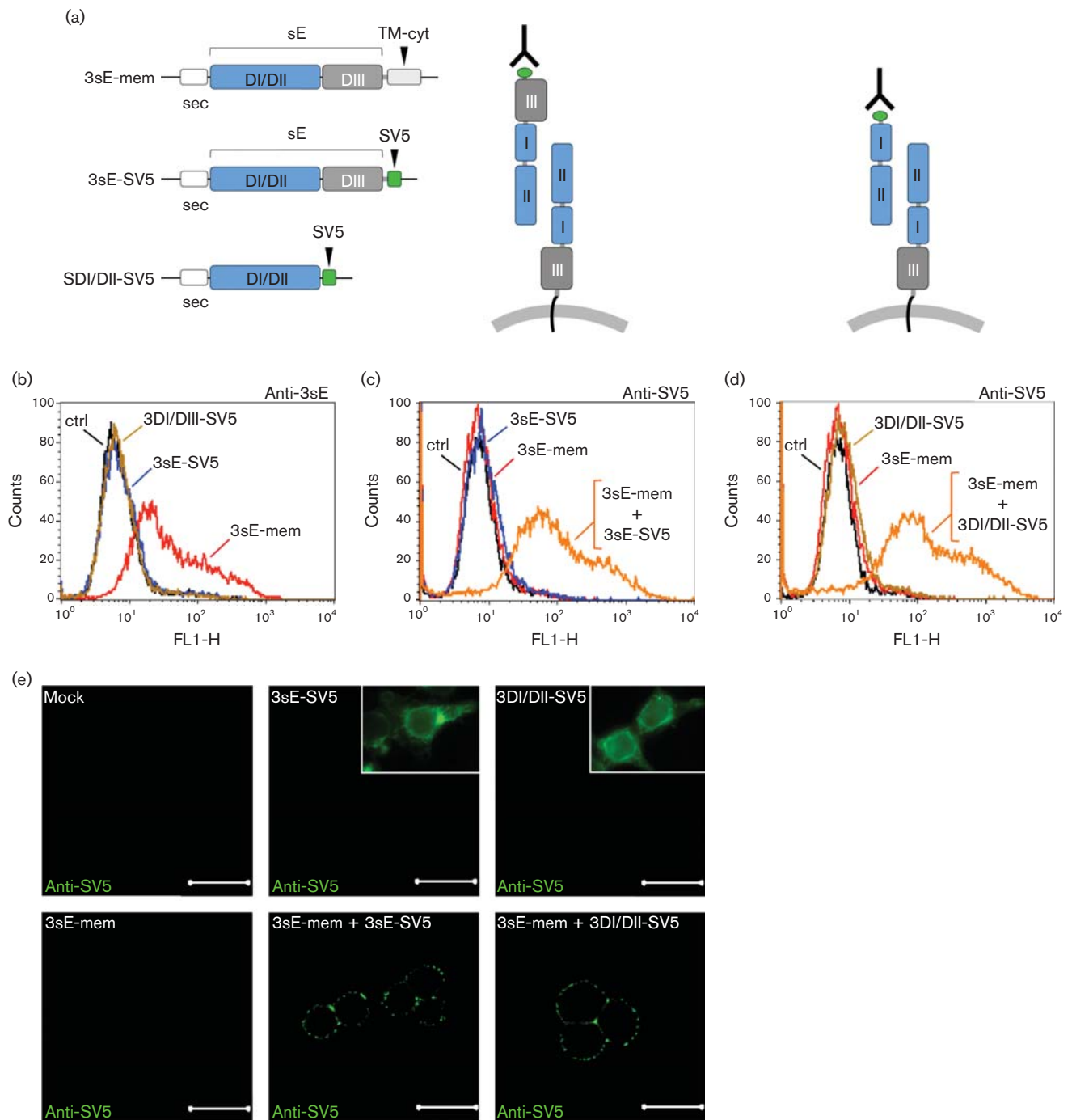


Fig. 4. Secretory sE and DI/DII proteins are able to form dimers. (a) Schematic of DNA constructs (left) and of the homo-dimers (sE-sE) and hetero-dimers (DI/DII-sE) detected on the cell membrane (right). (b-d) Cytofluorimetry plots of cells transfected with the indicated constructs and reacted with an anti-3sE serum (b), or with anti-SV5 to detect homo-dimers (c) and hetero-dimers (d). (e) Immunofluorescence of cells transfected with the indicated constructs and reacted with anti-SV5 to detect homo- and hetero-dimers. Permeabilized controls are shown as inserts within the respective images to highlight intracellular expression of the 3sE-SV5 and 3DI/DII-SV5 constructs. Bar, 20 μ m.

one produced at 37 °C, was recognized by 4G2, consistent with secretion being associated to proper DII folding (Fig. S3c). However, despite recognition by 4G2, 2sE

produced at 28 °C was still poorly secreted, thus indicating that other factors restrict its secretion in these conditions (Fig. S3c).

sE dimerization

We next tested the ability of the secreted sE and DI/DII to dimerize. We developed a cell-based assay to detect dimers by cytofluorimetry (Fig. 4a). 3sE was displayed on the cell membrane by fusing it to the trans-membrane and cytoplasmic domains of the human MHC-I α chain (3sE-mem) (Fig. 4b) and co-expressed with SV5-tagged secretory versions of 3sE or 3DI/DII. If secretory proteins were forming a hetero-dimer with the membrane-bound 3sE-mem, cells should stain positive for SV5. Indeed, cells became strongly positive for anti-SV5 only when co-transfected with constructs encoding the membrane-bound and secretory 3sE, but not when transfected with each construct alone (Fig. 4c). DI/DII-SV5 was also detected on the cell membrane when co-expressed with 3sE-mem (Fig. 4d) indicating that, despite the lack of DIII, DI/DII was still able to form a stable hetero-dimer with sE. These results were confirmed by immunofluorescence (Fig. 4e).

sE dimerization of the secretory 3sE, 4sE and the 2sE(3DII) chimera was further confirmed by co-immunoprecipitation. Cells were co-transfected with two sE constructs containing different C-terminal tags: SV5 or BAP (a 15 aa long

tag that is biotinylated *in vivo* by co-expression with an engineered biotin-ligase BirA active in the endoplasmic reticulum lumen; Predonzani *et al.*, 2008). Cell extracts were immunoprecipitated with anti-SV5 and analysed by WB with HRP-conjugated streptavidin (StrAv). All three sE were able to co-immunoprecipitate the BAP-tagged partner from both extracts (Fig. 5a) and supernatants (Fig. 5b). Although DI/DII from serotype 3 was found dimerized intracellularly (Fig. 5c), it was not similarly found in the supernatants (Fig. 5d), suggesting low stability of DI/DII dimers after secretion.

Indeed, DIII plays an important role in dimer stabilization. Secreted material in dimeric form was observed for 3sE homo-dimers (which contain two DIII) and for sE and DI/DII hetero-dimers (which contain a single DIII), but not for DI/DII homo-dimers (with no DIII) (Fig. 6a). In the case of sE and DI/DII hetero-dimers, the same result was obtained when pull-down was performed on sE or DI/DII (Fig. 6a, lanes 6 and 7). However, the amount of co-immunoprecipitated partner from hetero-dimers between sE and DI/DII was reduced around fourfold (approximately 5 %) compared with the sE homo-dimers

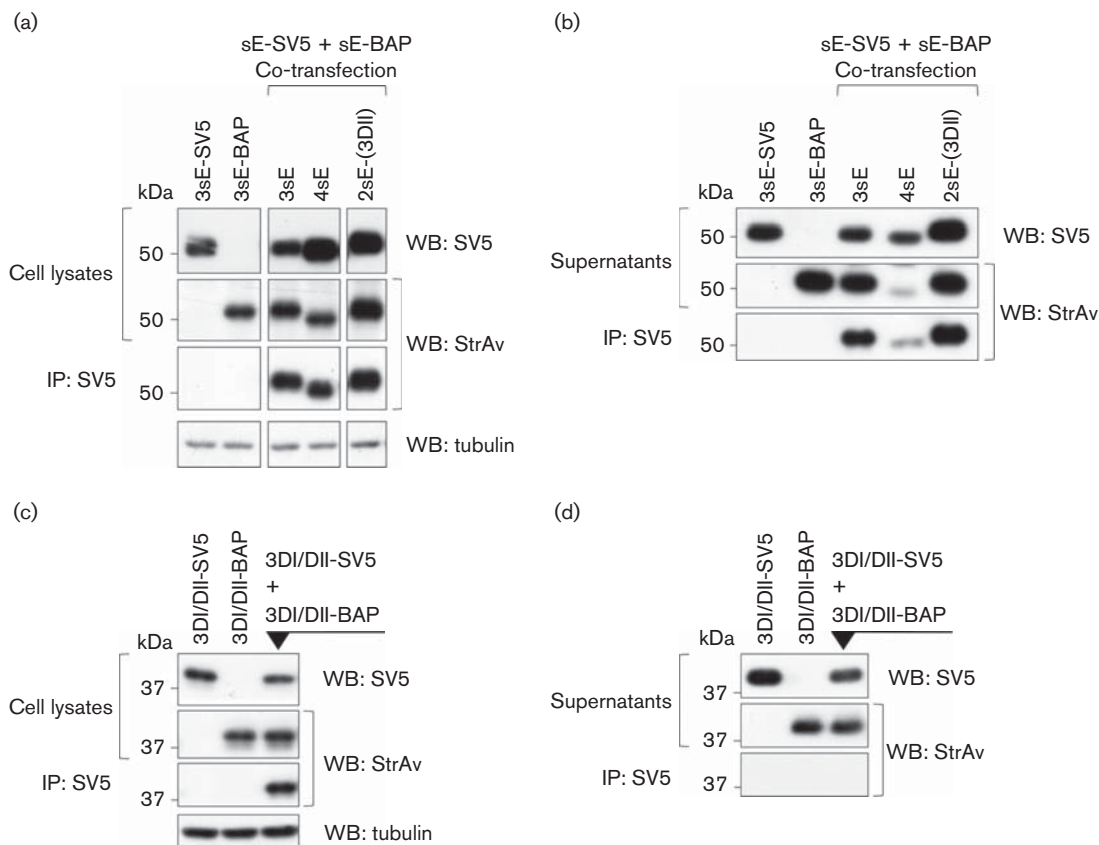


Fig. 5. Dimerization of secretory sE and DI/DII. Co-immunoprecipitations from cellular lysates (a, c) or supernatants (b, d) of HEK293T cells transfected with the indicated constructs. Immunoprecipitations were carried out with anti-SV5 and co-immunoprecipitated partners were detected with HRP-conjugated streptavidin (StrAv). In (a) and (c), anti-tubulin was used as a loading control.

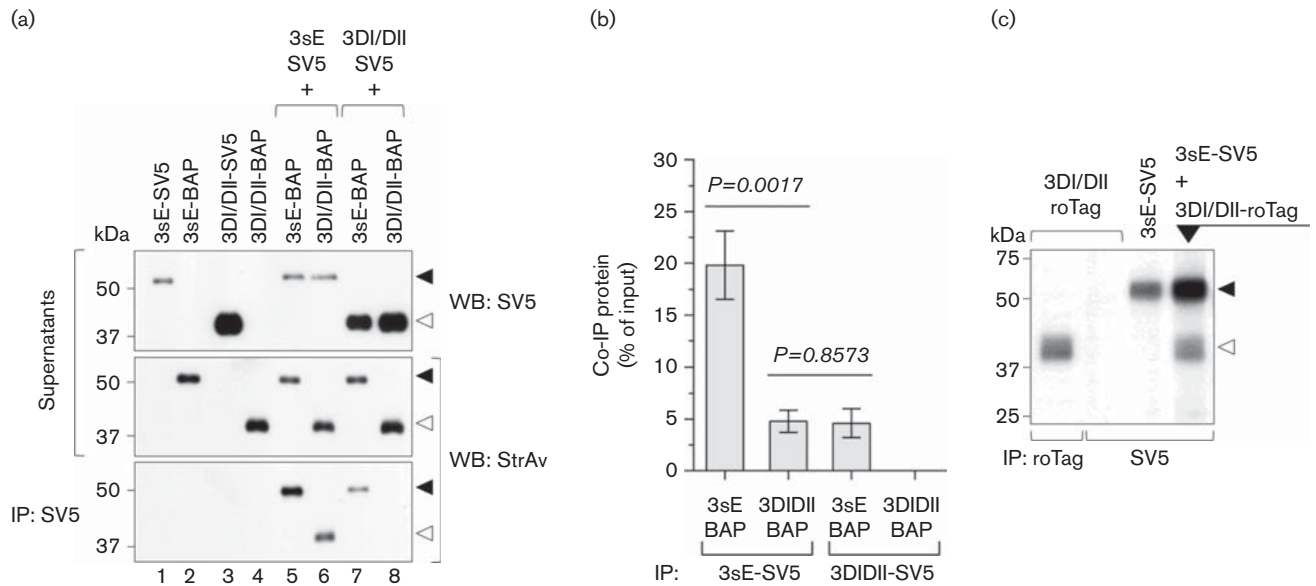


Fig. 6. Dimers are stabilized by DIII. (a) Co-immunoprecipitations of secreted material in supernatants of HEK293T cells transfected with serotype 3 constructs to analyse the formation of homo- and hetero-dimers. The amount of the anti-SV5 immunoprecipitated sample loaded into the gel (bottom panel) corresponds to ten times the amount of the input (middle panel). (b) Quantification of the data shown in (a), lanes 5–8. (Mean \pm SD of three independent experiments is shown.) (c) Immunoprecipitations with anti-SV5 or anti-roTag of cellular extracts from HEK293T cells transfected with the indicated constructs and [³⁵S]methionine-labelled (15 min pulse). Filled and open arrowheads correspond to sE and DI/DII, respectively.

(approximately 20 %), indicating that a single DIII stabilizes dimers to a lower extent than two of them (Fig. 6b). The specificity of this interaction was further confirmed by anti-SV5 immunoprecipitations of cell extracts from [³⁵S]methionine-labelled cells co-expressing 3sE (SV5-tagged) and 3DI/DII (roTag-tagged). 3DI/DII was co-immunoprecipitated with anti-SV5 only when co-expressed with 3sE (Fig. 6c).

Secretion and the immune response

It is expected that antigen secretion from transfected cells is an essential step for induction of antibody responses in DNA vaccines (Hon *et al.*, 2005; Shedlock & Weiner, 2000). Therefore, sE constructs with well-established secretory phenotypes would be preferred in order to optimize antigen availability and antibody responses as we have recently shown with DIII-based vaccines (Poggianella *et al.*, 2015). This hypothesis was confirmed by gene-gun DNA immunization of mice using two sets of constructs coding for sE with the same DIII but with different secretory phenotypes: (i) 3sE (secreted) and 3sE(2DII) (poorly secreted); and (ii) 2sE (poorly secreted) and 2sE(3DII) (secreted). Antibody titres against DIII were then determined by ELISA using recombinant DIII of serotypes 2 and 3 for coating. Mice immunized with the secretory 3sE construct had a titre against 3DIII above 2000, while those immunized with the poorly secretory 3sE(2DII) were negative at 1 : 100 dilution (Fig. 7a).

Cross-reactivity of 3sE sera against 2DIII was very poor (Fig. 7b). For constructs expressing 2DIII, the more efficiently secreted 2sE(3DII) induced a titre against 2DIII tenfold higher than the poorly secretory 2sE (Fig. 7c), while none of them showed significant reactivity against 3DIII (Fig. 7d). Antibody titres against the homologous DIII are summarized in Fig. 7(e) and underline the importance of antigen secretion and availability to induce strong antibody responses. The differences in responses to 2DIII and 3DIII are likely the consequence of the intrinsic immunogenicity of each antigen (Poggianella *et al.*, 2015). Immunofluorescence microscopy revealed that antibodies induced with 2sE, 2sE(3DII) and 3sE were able to recognize protein E in virus-infected cells (Fig. 7f), indicating reactivity against the mature viral protein.

Collectively, the data shown indicate that the secretory chimera 2sE(3DII) represents a valid construct to induce strong antibody responses against serotype 2 DIII upon DNA-immunization with sE constructs. Indeed, antibodies induced with 2sE(3DII) showed high neutralizing activity against serotype 2 (> 1 : 300) and much lower against serotype 3 (approximately 1 : 50) (Fig. 8a), a characteristic linked to the high specificity of serotype 2 anti-DIII responses (Poggianella *et al.*, 2015). As expected, antibodies induced with 2sE showed very low neutralizing activity against serotype 2 and no activity against serotype 3 (Fig. 8b), while those induced with 3sE neutralized serotype 3 but not serotype 2 (Fig. 8c). The poorly secretory

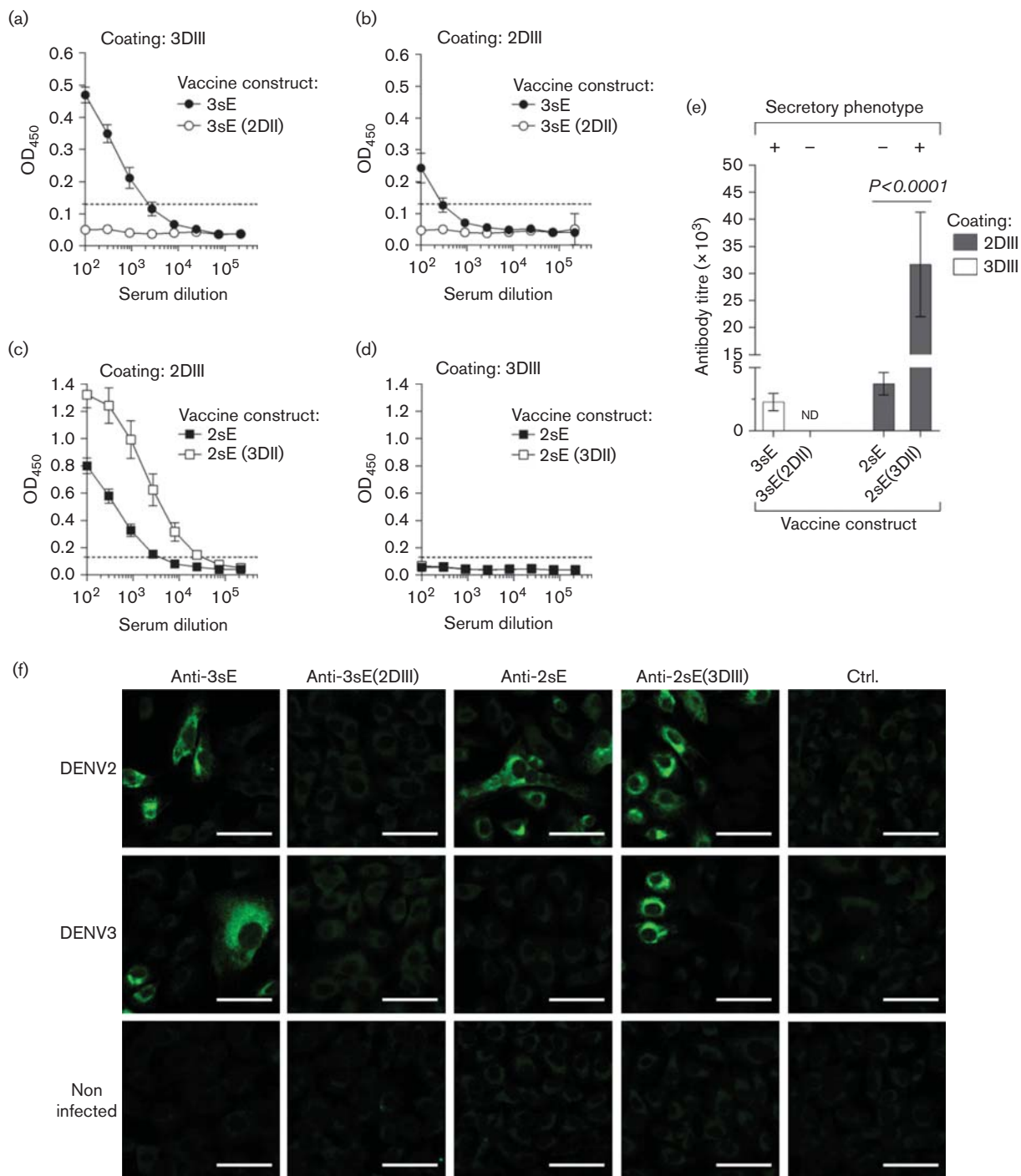


Fig. 7. Antibody responses against secretory and poorly secretory antigens upon DNA-vaccination. Anti-DIII ELISA of sera from mice gene gun-immunized with secretory and poorly secretory serotype 2 (a, b) or serotype 3 (c, d) constructs, tested on plates coated with 3DIII (a, d) or 2DIII (b, c), as indicated (in each case, mean \pm SD of four independent experiments done in triplicate is shown). (e) Plot of antibody titres obtained from the curves shown in (a) through (d). ND indicates an antibody titre below the cut-off value at 1 : 100 dilution. (f) Immunofluorescence of Vero cells non-infected or infected with DENV2 or DENV3 and reacted with sera from animals immunized with sE, 2sE(3DII), 3sE and 3sE(2DII), as indicated or the negative control sera (Ctrl.). Bars, 50 μ m.

chimera 3sE(2DII) did not have any neutralization activity (Fig. 8d) in agreement with the ELISA and immunofluorescence results. Antibody neutralization titres for the

homologous DIII serotype are summarized in Fig. 8(e). We then compared the avidity indexes of sera from mice immunized with constructs 2sE and 2sE(3DII) against

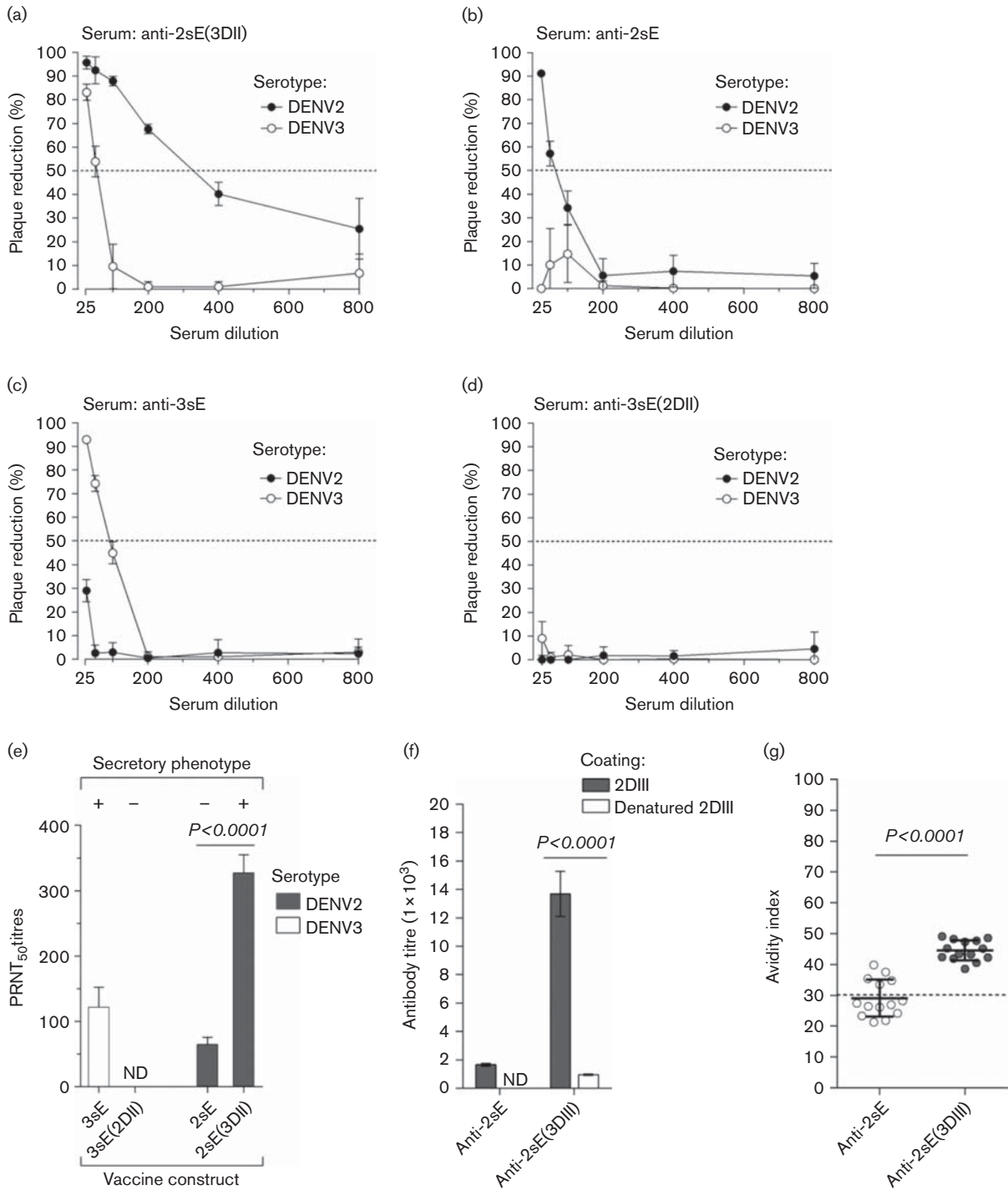


Fig. 8. Virus-neutralizing activity of secretory and poorly secretory antigens. (a–d) Plaque reduction neutralization test (PRNT) on DENV serotypes 2 (filled symbols) and 3 (open symbols) of pooled sera from groups of six animals immunized with 2sE(3DII) (a), 2sE (b), 3sE (c) and 3sE(2DII) (d) (in each case, mean \pm SD of three independent experiments done in duplicate is shown). (e) Summary of PRNT₅₀ titres of sera from the indicated secretory and non-secretory antigen constructs tested on the homologous DENV serotype. ND indicates a PRNT₅₀ titre below 25. (f) ELISA performed on equal amounts of native or denatured biotinylated 2DIII- ϵ CH4, captured on avidin-coated plates and reacted with sera from the indicated constructs (mean \pm SD of three independent experiments done in triplicate is shown). ND indicates an antibody titre below the cut-off value. (g) Avidity index of sera derived from animals immunized with 2sE or 2sE(3DII) determined on native 2DIII (in each case, mean \pm SD of fourteen independent measurements is shown).

2DIII. Interestingly, in addition to differences in antibody titres, and despite the fact that both sera are mainly directed towards conformational epitopes (Fig. 8f), antibodies derived from the secretory 2sE(3DIII) showed a significantly higher avidity index than those obtained from 2sE (Fig. 8g).

DISCUSSION

Although the use of DIII alone as an antigen for DENV vaccine development has been proved to induce antibodies against neutralizing E epitopes (Pierson *et al.*, 2008; Poggianella *et al.*, 2015), recent data have demonstrated the presence of broadly neutralizing antibodies directed against epitopes on DI and DII (Sukupolvi-Petty *et al.*, 2010; Wahala *et al.*, 2009; Williams *et al.*, 2012), and on E dimers (envelope dimer epitopes) that are reactive with all four serotypes (Dejnirattisai *et al.*, 2015; Rouvinski *et al.*, 2015).

Here we investigated the expression of E ectodomains from all DENV serotypes in mammalian cells to understand their secretory characteristics for implementation in DNA-based vaccines. We showed that efficient antigen secretion from transfected cells was required for an appropriate immune response in a DNA-immunization context.

Our data show that sE from different serotypes have distinct characteristics in relation to their secretory phenotype from mammalian cells. sE of serotypes 1 and 2 were not efficiently secreted, while those from serotypes 3 and 4 were. The poorly secretory phenotype of serotypes 1 and 2 was mapped to DII and was directly associated to the lack of proper DII folding, which in the case of serotype 2 was temperature dependent. Folding of DII was probed with mAb 4G2, which recognizes a conformational epitope comprising the highly conserved fusion loop that depends on several other loops and β -strands throughout DI/DII for proper folding and is instrumental for E dimerization (Allison *et al.*, 2001).

Despite mapping the sE secretory phenotype to DII, both DI and DII show high amino acid identity when comparing the poorly secretory with the secretory phenotypes (71 % for DI and 75 % for DII when comparing serotypes 2 and 3). Thus, a clear indication of possible DII residues that could explain the differences in folding and secretion was not apparent, suggesting that the compromised folding of the poorly secretory proteins probably involves cumulative effects of different residues distributed throughout the domain. It is unlikely, although not totally ruled out, that the secretory phenotypes described were dependent on the virus strains used since the E amino acid sequences are rather conserved within each serotype with an amino acid diversity equally distributed among the different domains (Fig. S4).

In virus-infected cells proper E folding and assembly into viral infective particles could be dependent on cellular

factors or viral proteins. For instance, the viral Pr-M protein has been described to assist E folding in a chaperone-like manner (Li *et al.*, 2008; Zheng *et al.*, 2010). However, the inclusion of Pr-M in subunit vaccines has been questioned, since recent evidence indicates that anti-Pr antibodies can enhance infection by promoting internalization of immature viruses (Dejnirattisai *et al.*, 2010; Rodenhuis-Zybert *et al.*, 2010). Also, the stem and anchor regions of E were shown to affect E expression in the absence of viral infection (Klein *et al.*, 2013).

Since most of neutralizing epitopes on E appear to be conformational in nature (Sukupolvi-Petty *et al.*, 2010), obtaining the E protein in its native conformation is critical for the development of an efficient vaccine against DENV (Tsai *et al.*, 2012). Several C-terminal truncated versions of E, in which the stem and the transmembrane anchor of the protein have been removed, have been developed for DNA and protein subunit immunizations (Coller *et al.*, 2011; Guzmán *et al.*, 2003; Mani *et al.*, 2013; Ocazionez Jimenez & Lopes da Fonseca, 2000). However, few studies have analysed production and secretion of such proteins from mammalian cells for DNA vaccination purposes. A previous study comparing the use of different full-length and truncated versions of E from DENV1 in the context of DNA vaccination (Raviprakash *et al.*, 2000) revealed that truncated proteins were poorly secreted and their secretion was not improved by co-expression with other viral proteins. Poor secretion of a DENV2 truncated ectodomain from BHK-21 cells was also recently shown to induce low neutralization titres in DNA vaccinated mice (Azevedo *et al.*, 2011), which resemble the anti-2sE responses shown here. Conversely, a recent work on a series of C-terminal truncated constructs of DENV4 E protein concluded that this protein could achieve proper folding and secretion by itself (Hsieh *et al.*, 2014; Tsai *et al.*, 2012). While these observations are in agreement with our data on secretory and poorly secretory phenotypes, contrasting data on the secretion analysis of sE from all serotypes in an AAV-based genetic vaccine model was recently reported, showing that only sE from DENV1 was secreted (Li *et al.*, 2012), indicating that some undefined factors may also play a role in secretion. Regardless of the secretory phenotype described by these studies, the available data further highlight the importance of detailed analysis of the antigen for genetic vaccines.

The recent description of E dimer-dependent epitopes located on a serotype-invariant site at the E-dimer interface (Rouvinski *et al.*, 2015) highlights the importance of ensuring proper E folding and dimerization in E-based immunogens, in particular for DNA vaccines. In addition, antigens presented in a dimeric structure were shown to have enhanced abilities to bind and activate relevant receptors on the surface of immune cells, including B-cells, which could be important for inducing stronger immune responses (Saenz *et al.*, 2014). When analysing dimerization properties of sE and DI/DII proteins, we found that secretory sE (serotypes 3 and 4) were capable of forming

stable dimers (approximately 20 % of secreted sE), in agreement with previous reports (Rouvinski *et al.*, 2015). Interestingly, dimer stability was found to be dependent on DIII, as secreted hetero-dimers between sE and DI/DII were less abundant than sE–sE homo-dimers, and those between DI/DII totally absent. This role of DIII in dimer stabilization has been previously suggested (Liao *et al.*, 2010; Zheng *et al.*, 2010).

Since the first human clinical trial for a DNA vaccine against human immunodeficiency virus (MacGregor *et al.*, 1998), several other candidates have been developed and tested in Phase 1 studies against a wide variety of infectious diseases (Graham *et al.*, 2006; Ledgerwood *et al.*, 2011; Martin *et al.*, 2006, 2007; Sarwar *et al.*, 2015). Although there are a number of studies describing DNA vaccines against dengue (Apt *et al.*, 2006; Azevedo *et al.*, 2011; Galula *et al.*, 2014; Khanam *et al.*, 2006; Konishi *et al.*, 2006; Li *et al.*, 2012; Ocazionez Jimenez & Lopes da Fonseca, 2000; Prompetchara *et al.*, 2014; Ramanathan *et al.*, 2009; Raviprakash *et al.*, 2001, 2006), to date there has been only one human clinical trial for a dengue DNA vaccine involving a Phase 1 study of a plasmid expressing the PrM and E proteins of DENV1 (Beckett *et al.*, 2011). In all cases, these vaccines have been proved to be safe and well-tolerated in humans, although low immunogenicity is still a concern associated with genetic vaccines in general (Coban *et al.*, 2011; Danko *et al.*, 2011). DNA immunization with constructs displaying the same antigenic determinants but different secretory capacity allowed us to demonstrate that antigen secretion is indeed an important characteristic to take into consideration for the design of efficient genetic vaccines. This could explain the poor immunological performances of many constructs so far tested (De Paula *et al.*, 2008; Konishi *et al.*, 2006; Lima *et al.*, 2011; Raviprakash *et al.*, 2000). We show that strategies based on chimerism could be used to enhance the antibody response against epitopes that are otherwise poorly secreted. For example, the response against DIII of serotype 2 was significantly improved in terms of antibody titres, neutralization and overall avidity with a chimera containing 3DII that conferred a secretory phenotype. Similarly, DI/DII chimeras can be used to promote secretion of otherwise non-secretory DI.

We have provided compelling evidence that proper design of E-based antigens is crucial to achieve efficient antibody responses against DENV following DNA immunization.

METHODS

Cell lines and viruses. HEK293, HEK293T/17, Vero, U-2OS and HeLa cells (ATCC, identifications CRL-1573, CRL-11268, CCL-81, HTB-96, CCL-2, respectively) were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10 % heat-inactivated FCS (Life Technologies), 50 µg ml⁻¹ gentamicin and

2 mM L-glutamine. Vero FM cells were maintained in the same conditions with 1 % non-essential amino acids. Cell cultures were grown at 37 °C (or 28 °C) with 5 % CO₂. DENV2 NGC strain and DENV3 3140/09 isolate were propagated in Vero (DENV2) or Vero FM cells (DENV3) and used for PRNT.

Plasmid DNA constructs. Sequences coding for the envelope ectodomains were obtained from strains: DENV1 Nauru Island, DENV2 New Guinea C, DENV3 H87 and DENV4 Dominica (GenBank accession numbers U88535.1; AF038403; M93130; AF326573). Codon optimized sE sequences of all DENV serotypes were obtained as synthetic fragments from GenScript. Each sE sequence was fused to an immunoglobulin leader sequence (sec) at the N terminus (Li *et al.*, 1997) and the SV5 tag (GKPIPPLLGLD) (Hanke *et al.*, 1992) at the C terminus, and cloned into a pcDNA3 vector (sE-SV5). The DI/DII-SV5 and DIII-SV5 constructs were derived by deleting DIII or DI/DII coding regions, respectively (Fig. S1). Chimeric constructs were obtained as synthetic fragments from GenScript. The sE, DI/DII or chimeric constructs tagged with BAP (biotin acceptor peptide, GLNDIFEAQKIEWHE) or roTag were obtained by cloning into the corresponding vectors as previously reported (Beckett *et al.*, 1999; Petris *et al.*, 2014; Predonzani *et al.*, 2008). 3sE-mem construct was derived from the 3sE-SV5 plasmid after replacing the SV5 tag with the transmembrane and cytoplasmic domains of the human MHC-I α chain.

Expression of recombinant dengue molecules. Transfections of HEK293T/17 cells were performed with the standard calcium phosphate method (Sambrook *et al.*, 1989), while transfections of HeLa, U-2OS and Vero cells were performed using Lipofectamine LTX (Life Technologies). Cellular extracts were prepared in 100 µl of TNN lysis buffer (100 mM Tris/HCl, pH 8, 250 mM NaCl, 0.5 % NP-40) at 4 °C, supplemented with Protease Inhibitor Cocktail (Sigma-Aldrich). Recombinant biotinylated DIII- ϵ CH4-BAP from DV2 and DV3 were obtained as previously reported (Poggianella *et al.*, 2015).

Antibodies and reagents. HRP-conjugated goat anti-mouse IgG, HRP-conjugated streptavidin and Alexa488-conjugated goat anti-mouse IgG were purchased from Jackson ImmunoResearch. mAb anti-tubulin (clone DM1A) and mAb 4G2 were from Millipore and HRP-conjugated mAb anti-actin (clone AC-15) was from Sigma-Aldrich.

Western blot and slot blot analyses. Samples were separated by 10 % SDS-PAGE, transferred onto PVDF membranes (Millipore) and probed with anti-SV5 (1 : 10 000) followed by HRP-linked goat anti-mouse IgG. For biotinylated proteins, membranes were incubated with HRP-linked streptavidin (1 : 20 000). Signals were visualized by ECL (Thermo-Pierce). Unless otherwise indicated, equivalent amounts of cellular extracts and culture supernatant samples were used in each experiment. For slot blots, normalized samples were blotted onto PVDF membranes using the Bio-Dot SF Apparatus (Bio-Rad). Denatured samples were boiled with 2 % mercaptoethanol (Sigma-Aldrich) before blotting. Incubation with anti-SV5 or 4G2 (1 : 1500) was followed by HRP-conjugated goat anti-mouse IgG (1 : 5000).

Co-immunoprecipitations. Immunoprecipitations with anti-SV5 (1 : 500) and protein A agarose (Repligen) were performed for 2h at 4 °C, loaded onto Micro Bio-spin columns (Bio-Rad) and washed with TNN buffer followed by RIPA buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.5 % sodium deoxycholate, 0.1 % SDS, 1 % NP-40, 1 mM EDTA). SDS-PAGE sample buffer was used for elution. Bands were quantified using ImageJ 1.47v software (National Institute of Health).

Radioactive labelling. Transfected cells were incubated for 30 min in DMEM without L-methionine and L-cysteine, supplemented with dialysed FCS. A [³⁵S]-methionine and [³⁵S]-cysteine mix (Expre³⁵S; Perkin Elmer) was added (200 µCi ml⁻¹) and incubated for 15 min. Cells were then lysed in TNN buffer and cell extracts immunoprecipitated for 2 h at 4 °C with anti-SV5 antibody and Protein A-agarose. Immunoprecipitated proteins were eluted from agarose beads with reducing SDS-PAGE sample buffer, separated on 10 % SDS-PAGE, and developed by autoradiography on Kodak BioMax XAR films (Carestream Health).

Animal immunizations. Groups of six female BALB/c mice of 5–6 weeks old (Harlan) were immunized three times at 15 day intervals (days 1, 15 and 30) by biolistic delivery of 1 µm gold particles coated with 1 µg of DNA using Gene Gun technology (Bio-Rad) (Benvenuti & Burrone, 2001; Cesco-Gaspere *et al.*, 2005); blood samples were collected at days 45 and 60 by submandibular puncture. All animal procedures were performed in compliance to laws and policies established in the legislation (D. L.vo 26/2014 of the Italian Government, protocol DGSAF0024706).

Cytofluorimetry and immunofluorescence. For cytofluorimetry, cells were washed and resuspended in PBS containing 3 % BSA and 5 µM EDTA, incubated with anti-SV5 (1 : 2000) for 1 h at room temperature followed by Alexa488-conjugated goat anti-mouse IgG (1 : 1000) and analysed in a FACSCalibur (BD Biosciences). For immunofluorescence, transfected HEK293T cells plated on polylysine (Sigma-Aldrich) coated slides, or Vero cells infected with DENV2 and DENV3 (m.o.i. of 0.1) for 36 h, were fixed with 3.7 % paraformaldehyde in PBS, quenched with 100 mM PBS glycine and incubated with anti-SV5 (1 : 2000) or anti-2sE, anti-2sE(3DII), anti-3sE and anti-3sE(2DII) and control sera (1 : 100) followed by Alexa488-conjugated goat anti-mouse IgG (1 : 1000). For permeabilization, cells were treated with 1 % Triton (Sigma-Aldrich) in PBS. Images were acquired using a Zeiss LSM510 confocal microscope.

ELISA and avidity assay. ELISA IgG titres were determined as previously described (Poggianella *et al.*, 2015) and expressed as the reciprocal of the dilution at which the OD₄₅₀ was three times higher than that of the control serum (pre-immune serum or serum from animals immunized with empty vector). The avidity assay was carried out as previously described (de Souza *et al.*, 2004; Zompi *et al.*, 2012) and indexes were calculated as the ratio between the OD₄₅₀ obtained following washings with and without 6M urea, multiplied by 100.

Plaque reduction neutralization test (PRNT). PRNT assays were carried out as previously described (Poggianella *et al.*, 2015). Briefly, de-complemented mouse sera samples were twofold serially diluted and incubated for 1.5 h at 36 °C with an equal volume of DMEM containing 50 p.f.u. of dengue virus. Vero cells were infected in duplicate for 1 h at 36 °C; the viral inoculum was then removed and cells incubated at 36 °C for 7 days. After fixing (paraformaldehyde 3.7 %) and staining with 1 % crystal violet for 30 min, plaques were counted and the percentage of plaque reduction against control serum was calculated. Neutralizing antibody titres were expressed as the serum dilution yielding a 50 % plaque number reduction (PRNT₅₀).

Statistical analysis. All data shown were calculated from at least four independent experiments performed in duplicate or triplicate. All data are shown as arithmetic means ± SD and were analysed using GraphPad Prism (version 6.0) software (GraphPad Software). Unpaired two-tailed *t*-test and one-way ANOVA test were used to analyse the data; *P* values < 0.05 were considered significant in both cases.

ACKNOWLEDGEMENTS

J. L. S. C. was supported by an International Centre for Genetic Engineering and Biotechnology (ICGEB) pre-doctoral fellowship. We would like to remember Mauro Sturnega, and thank Stefano Artico and the personnel of ICGEB Animal Experimentation Facility for their excellent technical assistance throughout the project.

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