Involvement of the Fcγ receptor IIA cytoplasmic domain in antibody-dependent enhancement of dengue virus infection

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Sub-neutralizing concentrations of antibody to dengue virus (DENV) enhance DENV infection of Fcγ receptor-expressing cells. This phenomenon, referred to as antibody-dependent enhancement (ADE), has been hypothesized to be responsible for the severe form of DENV infection, including dengue haemorrhagic fever and dengue shock syndrome. To analyse further the mechanisms of ADE in vitro, this study introduced a series of cytoplasmic mutants into human FcγRIIA. The mutated FcγRIIA was then expressed on COS-7 cells to see whether these mutants could enhance DENV infection. Wild-type FcγRIIA enhanced DENV infection, consistent with previous reports using FcγR-positive monocytes. Disruption of the immune tyrosine activation motif (ITAM) in the cytoplasmic domain of FcγRIIA or removing the sequences between the two ITAM regions eliminated ADE. These findings suggest that the specific structure of the FcγRIIA cytoplasmic domain is essential for the ability of FcγRIIA to mediate ADE.

INTRODUCTION

Dengue fever ranks as the most important mosquito-borne virus disease in the world, and an estimated 2.5 billion people live in over 100 endemic countries and areas where dengue viruses (DENVs) can be transmitted. With up to 100 million infections occurring annually, dengue fever is a source of considerable economic loss to health authorities (Okanurak et al., 1997), as well as to patients (Anderson et al., 2007).

DENV exists in four distinct viral serotypes (DENV-1 to -4), and each serotype can cause a spectrum of symptoms, ranging from mild febrile illness to severe life-threatening dengue haemorrhagic fever. Epidemiological studies have indicated that infection with a DENV serotype offers lifelong protection against homologous infection by the same serotype. Immunity generated against a particular dengue serotype does not provide protection towards infection with a previously unexposed serotype in the long term. Instead, after a short period of cross-protection against heterologous serotypes, antibodies generated against primary DENV infection are postulated to be one of the main factors contributing to the severe form of DENV infection (Graham et al., 1999; Sangkawibha et al., 1984; Vaughn et al., 2000). Under the conditions of anti-DENV antibody cross-reactivity or at concentrations where neutralization does not occur, virus–antibody complexes are taken up more readily than uncoated virus particles by cells expressing Fcγ receptors (FcγRs), such as monocytes and macrophages (Konny et al., 1988; Littaua et al., 1990). This effect, known as antibody-dependent enhancement (ADE), has been demonstrated for both RNA and DNA viruses, and has been studied extensively with DENV (Littaua et al., 1990; Schlesinger & Chapman, 1999).

Interaction of FcγR and antigen–antibody complexes triggers an array of responses, which include phagocytosis, endocytosis, antibody-dependent cell-mediated cytotoxicity, superoxide generation and release of inflammatory mediators, as well as immune-complex clearance (An, 1982; Gessner et al., 1998; Indik et al., 1991, 1995a, b; Mero et al., 2006; Ravetch & Kinet, 1991). These responses are largely dependent upon interactions between FcγR and protein, and lipid signalling transduction moieties located in close proximity to the cytoplasmic and transmembrane regions of FcγR (Barabé et al., 2002; Booth et al., 2002; García-García & Rosales, 2002). Endocytosis of opsonized particles by FcγRIIA (CD32A) involves lipid raft–induced receptor clustering, which leads to signalling through the Ig family gene tyrosine activation motif (ITAM: E-X8-D-X2-YXXL-X12-YXXL), observed in the cytoplasmic domains of several Ig gene family receptors (Abdel Shakor et al., 2004; Huang et al., 1992; Indik et al., 1991; Kwiatkowska et al., 2003; Sobota et al., 2005). When mutations are introduced into the tyrosine moieties located in the ITAM region of
FcγRIIA, kinase-mediated phagocytosis of opsonized particles is severely reduced (Kim et al., 2003; Mitchell et al., 1994). In contrast, the ability of FcγR to associate with lipid rafts by substitution of a cysteine residue within the juxtamembrane region (Barnes et al., 2006). Transmembrane and cytoplasmic structures are thus required for FcγR functions and are likely to be involved in interactions with intermediate signal transduction elements that are components in the immune-complex internalization machinery.

However, the importance of such internalization machinery in FcγR-mediated ADE remains obscure. FcγR could facilitate the entry of DENV by directing the virus to the cell surface and, in turn, increasing the probability of interactions between DENV and its unidentified viral receptor (Mady et al., 1991). In contrast, signalling systems triggered by FcγR may lead to internalization of the viral DENV–antibody immune complex and thus enhanced infection.

In the present study, we evaluated the possible roles of the cytoplasmic and transmembrane regions of FcγRIIA in facilitating DENV infection in the presence of antibody. We introduced a series of mutations in the cytoplasmic domains of wild-type (WT) FcγRIIA and examined the capacity for ADE. The cytoplasmic domain of the receptor, including its palmitoylation site (Barnes et al., 2006), was found to be required for ADE of DENV infection. The results indicate that FcγRIIA-mediated signal transduction is necessary for ADE.

RESULTS

Preparation of FcγRIIA receptor containing mutations in signalling domains

It has been reported that FcγRIIA mediates ADE using K562 cells, which express only FcγRIIA (Littaua et al., 1990). To define the requirement for the cytoplasmic domain in FcγRIIA-mediated ADE, the receptor with or without mutations was transfected into COS-7 cells, which lack endogenous FcγR (Indik et al., 1991). The FcγRIIA cytoplasmic region tyrosine residues (Y281, Y288 and Y304), designated P1, P2 and P3, respectively, contribute to the ability of receptors to undergo phagocytosis and capping (Kwiatkowska et al., 2003; Mitchell et al., 1994). The cysteine residue within the juxtamembrane region of FcγRIIA (C241) is involved in raft localization of FcγRIIA and efficient receptor signalling (Barnes et al., 2006). We introduced a series of point and deletion mutations of residues in the cytoplasmic domain of FcγRIIA that are involved in receptor signalling and phagocytosis of immune complexes (Fig. 1a, Table 1). The expression of each of the constructs in COS-7 cells was verified by immunoblotting (data not shown) and flow cytometry (Fig. 1b, Table 1). More than 50% of the transfected cells constantly expressed mutant and WT FcγRIIA, except for the mutants dT (48 ± 5%) and Y3F (34 ± 10%).

Phagocytic activities of COS-7 cells expressing mutated FcγRIIA

To confirm that the WT and mutated FcγRIIA maintained the biological function of the receptors, we first measured phagocytic activity. Phagocytic activity is the best-studied biological function of FcγRIIA (Indik et al., 1995a, b; Mitchell et al., 1994). We adopted a quantitative fluorescence method that employed anti-Escherichia coli polyclonal antibody (pAb)-opsonized, succinimidyl ester (SE)-labelled E. coli immunocomplex particles (Fig. 2a). With anti-E. coli pAb, COS-7 cells expressing WT, dT and dP3 exhibited higher levels of phagocytic activity (13.8 ± 2.9%, 13.6 ± 2.6% and 7.7 ± 1.5%, respectively) than those expressing the other FcγRIIA mutants or those without FcγRIIA (Fig. 2b). Less than 5% of COS-7 cells expressing WT, dT and dP3 were phagocytic when exposed to SE-labelled E. coli strain K-12 without anti-E. coli pAb (Fig. 2b). The results suggested that the phagocytic activity of WT-, dT- and dP3-expressing cells is FcγR-dependent and that the transfected FcγRIIA is functional.

Receptor clustering induced by binding of DENV–antibody complex to FcγRIIA

The consequences of the binding of DENV–antibody complexes to WT and mutant FcγRIIA were examined. The occurrence of cross-linking and capping was monitored by immunofluorescence as described in Methods. Capping occurred only on COS-7 cells that expressed WT, dT and dP3 (Fig. 3). Cross-linking, but not capping, occurred on cells expressing dP2, dP1P2, dP2P3, dP1P2P3, CT, dISR, Y3F and C241A (Fig. 3). Neither cross-linking nor capping occurred in WT-transfected COS-7 cells after inoculation with DENV-1 in the absence of antibody (Fig. 3, WT−AB). The results indicated that the ability of FcγRIIA to cluster after the binding of DENV–antibody complex varies depending on the induced mutations.

ADE of DENV infection in COS-7 cells expressing mutant FcγRIIA

Human serum from a DENV-3-infected patient was used to prepare DENV-1– or DENV-2–antibody complexes to examine ADE. COS-7 cells that expressed WT were infected with the human serum–DENV-1 or –DENV-2 complex and the cells were stained with dengue serotype-cross-reactive mAb 4G2 and examined by flow cytometry. The results indicated that the human anti-DENV serum enhanced DENV-1 and DENV-2 infection of WT-expressing COS-7 cells to the maximum levels at a final dilution of 1:1000 and 1:10000, respectively (Fig. 4). Based on these results, the serum was used at a 1:1000 dilution for DENV-1 and a 1:10000 dilution for DENV-2 in the following experiments.

To evaluate the incubation period of DENV-infected cells, COS-7 cells transfected with WT FcγRIIA were infected with DENV-1 with or without human serum treatment,
and the presence of DENV antigen-positive cells was examined by flow cytometry at different time points. The proportions of infected cells after DENV-1 infection with or without antibody were $0.4 \pm 0.3$ and $0.3 \pm 0.03\%$ at 48 h, $4.0 \pm 1.5$ and $0.6 \pm 0.2\%$ at 72 h, $12.2 \pm 6.0$ and $3.3 \pm 2.6\%$ at 96 h, and $15.9 \pm 6.9$ and $3.7 \pm 2.2\%$ at 120 h, respectively. The percentage of infected cells increased rapidly after DENV-1 infection with enhancing antibody, suggesting that the cells infected with DENV-1–antibody complex released progeny virus as efficiently as the cells infected with DENV-1 alone. We used the incubation period of 72 h after inoculation to assay the enhancement of DENV infection in primary infection.

**Fig. 1.** Structure of the mutated FcγRIIA constructs, and expression in COS-7 cells as determined by flow cytometry. (a) The mutants were constructed by standard site-directed mutagenesis methods, introduced into pcDNA3.1(+) and expressed in COS-7 cells. The FcγRIIA gene is shown in the figure with the extracellular region, transmembrane region (TM) and cytoplasmic region (CY) indicated. The FcγRIIA cDNA is numbered starting from +1. The filled box represents a hydrophobic stretch of 24 aa presumed to span the membrane. Lines indicate deleted regions, and the letters A and F represent substituted amino acids in the gene. (b) The percentage of COS-7 cells expressing WT and mutated FcγRIIA was determined by flow cytometry, using PE-labelled mAb 16320 against human FcγRIIA. Results are representative of four or more experiments performed in triplicate.
**Table 1.** Characteristics of the mutated FcγRIIA prepared in this study

<table>
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<tr>
<th>Mutant</th>
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<th>Positive cells (%)*</th>
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<td>dT</td>
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<td>dISR†</td>
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<tr>
<td>Y3F‡</td>
<td>Y304F</td>
<td>Y288F</td>
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<tr>
<td>C241A§</td>
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*Surface expression of WT and mutated FcγRIIA was examined by flow cytometry. Results are shown as means ± SD of three experiments performed in triplicate.
†Deletion of 12 aa located between ITAM1 and ITAM2.
‡A phenylalanine residue was substituted for tyrosine at aa 281, 288 and 304.
§An alanine residue was substituted for cysteine at aa 241.

DENV-1 and DENV-2 were mixed with anti-dengue human serum at a 1:1000 dilution and 1:10 000 dilution, respectively, and the DENV–antibody complex was prepared. COS-7 cells expressing WT or mutant FcγRIIA were inoculated with the DENV–antibody immune complex or DENV. The presence of infected cells was confirmed by an immunofluorescence assay (Fig. 5a), and the percentage of DENV antigen-positive cells was measured by flow cytometry (Fig. 5b, c). The percentage of antigen-positive cells was compared between transfected COS-7 cells infected with the DENV-1–antibody immune complex and those infected with DENV-1 alone. ADE of DENV-1 was detected in COS-7 cells expressing WT (11.2 ± 4.5 % after infection with immune complex and 11.0 ± 8.8 % after infection with virus alone), dT (9.5 ± 1.8 and 1.2 ± 0.4 %) and dP3 (4.7 ± 1.0 and 1.2 ± 0.3 %), but not in those expressing the other mutants of FcγRIIA: dP2 (1.3 ± 1.1 and 1.2 ± 1.4 %), dP1P2 (0.9 ± 1.2 and 1.5 ± 1.5 %), dP2P3 (0.9 ± 0.8 and 0.6 ± 0.1 %), dP1P2P3 (1.3 ± 0.7 and 1.8 ± 0.4 %), CT (1.5 ± 0.7 and 1.8 ± 1.4 %), dISR (1.2 ± 0.9 and 0.9 ± 0.3 %), Y3F (2.1 ± 0.7 and 1.0 ± 0.4 %), C241A (2.4 ± 1.3 and 2.1 ± 1.0 %) and mock transfected (1.1 ± 0.6 and 1.3 ± 0.5 %) (Fig. 5b). ADE of DENV-2–antibody immune complexes was also detected in cells expressing WT (34.1 ± 13.9 and 3.5 ± 0.9 %), dT (34.6 ± 5.3 and 4.4 ± 2.1 %), and dP3 (12.0 ± 0.3 and 2.2 ± 0.2 %), but not in cells expressing the other mutants: dP2 (2.2 ± 0.4 and 3.8 ± 1.0 %), dP1P2 (2.4 ± 1.2 and 1.3 ± 0.7 %), dP2P3 (2.2 ± 0.4 and 3.4 ± 1.6 %), dP1P2P3 (1.3 ± 0.5 and 2.3 ± 0.9 %), CT (2.5 ± 1.6 and 1.6 ± 0.8 %), dISR (2.4 ± 1.6 and 2.1 ± 1.1 %), Y3F (2.1 ± 0.7 and 1.1 ± 0.4 %), C241A (2.6 ± 0.5 and 2.0 ± 1.4 %) and mock transfected (2.4 ± 1.3 and 1.8 ± 0.6 %) (Fig. 5c). The results indicated that disruption of the ITAM motifs and removal of the sequences between the two ITAM motifs eliminated the ability of FcγRIIA to mediate ADE. The results thus suggest that the specific structure of FcγRIIA, and signal transduction via FcγRIIA, are both required for ADE during dengue virus infection.

**DISCUSSION**

Following entry of DENV into the bloodstream, the virus enters a target cell where it replicates, after which it can exist in several forms based on the level of viraemia and host response to the viraemia (Noisakran & Perng, 2008). Antibody response is an important defence mechanism employed to control DENV infection. Anti-DENV antibodies at sub-neutralizing concentrations, however, enhance DENV infection via FcγR (Kontny et al., 1988) and FcγRII (Littaua et al., 1990). DENV-infected cells in turn stimulate specific T lymphocytes, resulting in a rapid increase in inflammatory mediators. The mediators generated as a result of immune responses contribute towards progression of severe DENV infection, causing plasma leakage, shock and haemorrhagic manifestations (Kurane & Ennis, 1992). Circulation DENV immune complexes have been observed in 80% of dengue haemorrhagic fever cases (Ruangirachuporn et al., 1979). The ability of DENV to utilize FcγR for cell entry relies on the formation of a virus–antibody complex. Thus, identification of the early steps of interactions between the DENV–antibody complex and FcγR is important in elucidation of the mechanism of ADE.

FcγRIIA-transfected COS cells have proved useful for determining the functions of FcγR in mediating receptor tyrosine phosphorylation, phagocytosis (Mitchell et al., 1994) and endocytosis, when 15–30% of the cells expressed the transfected receptors (Davis et al., 1995). Specific structures of the transmembrane and cytoplasmic domain account for the ability of FcγRIIA to stimulate phagocytosis and tyrosine phosphorylation (Barnes et al., 1992; García & Rosales, 2002; Mitchell et al., 1994). Receptor phosphorylation is catalysed by rafts (Kwiatkowska et al., 2003), triggering signal pathways that target actin-based cytoskeleton reorganization, and this in turn serves as a driving force for FcγRIIA-mediated phagocytosis and FcγRIIA capping (Kwiatkowska et al., 2003). In order for the receptor to form, FcγR needs to cross-link, which in turn triggers FcγR clustering and receptor phosphorylation (Huang et al., 1992). Tyrosine phosphorylation of FcγR and accompanying proteins facilitates clustering of FcγR, thereby permitting efficient binding of particles and immune complexes (Sobota et al., 2005). These findings...
indicate that specific structures of FcγRIIA are crucial for triggering receptor-mediated signalling pathways and biological functions.

The present study was undertaken to determine whether modification of the conserved motifs of the cytoplasmic region of FcγRIIA affects the ability of the receptor to mediate ADE. The specificity of FcγRIIA-mediated phagocytosis and receptor capping was confirmed by experiments in the absence of enhancing or opsonizing antibodies, and by using COS-7 cells without FcγRIIA. Consistent with previous findings (Kwiatkowska et al.,

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**Fig. 2.** Phagocytosis of opsonized SE-labelled E. coli K-12 by COS-7 cells expressing mutant and WT FcγRIIA. (a) COS-7 cells expressing WT or mutated FcγRIIA were incubated with SE-labelled E. coli K-12 particles sensitized with anti-E. coli pAb at 37 °C for 45 min. Mock-transfected COS-7 cells served as controls. Phagocytosis of E. coli by COS-7 cells was observed by fluorescence microscopy. Bar, 20 μm. (b) The percentage of cells (mean ± SD) that phagocytosed E. coli was determined by flow cytometry. Opsinization (OP): the presence (+) or absence (−) of anti-E. coli pAb is indicated. *P<0.05.

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**Fig. 3.** Cross-linking and capping of anti-dengue serum–DENV-1 complexes on COS-7 cells expressing WT and mutated FcγRIIA. COS-7 transfectants were monitored for their ability to form cap-like structures following binding of the DENV–antibody complex. Capping of cross-linked cells was observed 10 min after warming at 20 °C. Each transfectant was stained with PE-labelled anti-hFcγRIIA (mAb 16320). WT−Ab represents cells stimulated with DENV in the absence of antibody. Arrows indicate capping, whilst arrowheads indicate cross-linking. Bar, 5 μm.
The results suggest that the structure of the ITAM region and removal of sequences between the ITAM motifs eliminated phagocytosis and the ability of FcγRIIA to undergo capping, indicating that the specific structure of FcγRIIA is required for ADE. To examine whether phagocytosis and DENV immune complex enhancement require similar signal transduction mechanisms, we performed a linear regression analysis and found a highly significant correlation (P<0.01) between the two processes (Fig. 6). The results suggest that the structure of the ITAM motif of FcγRIIA is, in part, involved in both ADE and phagocytosis. Signal transduction was not analysed in the present study, and further studies are needed to determine whether similar signal transduction mechanisms are involved in ADE and phagocytosis.

Although the cross-linking of receptor was observed by using anti-DENV serum in COS-7 cells transfected with Y3F, which carries mutations in the ITAM tyrosine moiety, capping, receptor-induced phagocytosis and enhancement of both DENV-1 and DENV-2 infection by anti-DENV serum were absent from FcγRIIA/Y3F. These results differ from the conclusion of a recent study, which observed that tyrosine residues in the ITAM region do not play a role in FcγRIIA-mediated ADE (Rodrigo et al., 2006). Differences in the transfection and infection methods, as well as variations in antibodies and virus strain, may be the reason for the different results, but this should be investigated further.

We conclude that the specific structure of FcγRIIA, when present on non-professional phagocytic cells, is crucial for mediating processes that promote ADE. The results provide a profound implication for our understanding of the mechanism of DENV entry into cells in the presence of antibody.

**METHODS**

**Cell lines.** COS-7 cells, an African green monkey kidney-derived fibroblast cell line, and Vero cells (ATCC CCL-81), an African green monkey kidney-derived epithelial cell line, were used. Cells were cultured in Eagle’s minimum essential medium (EMEM; Sigma), supplemented with heat-inactivated 10% fetal calf serum (FCS; Sigma) without antibiotics at 37 °C in 5% CO₂.

**Virus and antibodies.** DENV-1 strain 01-44-1HuNIID (GenBank accession no. AB111070), isolated from Tahiti in 2001 (Ito et al., 2007), and DENV-2 strain D2/Hu/OPD30NIID/2005 (TL-30) (GenBank accession no. AB219135), isolated from East Timor in 2005, were used. Virus was propagated on Vero cells. Titres of DENV were determined by plaque assay in Vero cells. Virus dilutions in volumes of 100 μl were inoculated on Vero cell monolayers in 12-well plates. The plates were incubated for 60 min at 37 °C in 5% CO₂. After virus adsorption, the cells were overlaid with maintenance medium containing 1% methylcellulose (Wako Pure Chemical Industries). The plates were incubated at 37 °C in 5% CO₂ for 5 days and then fixed with neutral formalin for 60 min at room temperature. The cells were then stained with 0.3% methylene blue for 60 min at room temperature and washed with tap water. Plaques were counted and the virus infectivity titre was expressed as p.f.u. ml⁻¹. Human serum from a dengue fever patient caused by DENV-3 was used as the enhancing antibody. Dengue serotype-cross-reactive mouse IgG monoclonal antibody (mAb) 4G2 (ATCC HB-112), which recognizes the E protein, was used in immunofluorescent and flow cytometry assays.

**FcγRIIA and mutant FcγRIIA plasmid constructions.** Human FcγRIIA cDNA (Brooks et al., 1989, GenBank accession no. M31932) was generously provided by Dr Jeffrey V. Ravetch, Rockefeller University, NY, USA. The cDNA was subcloned into pcDNA3.1/neo+ (Invitrogen) and mutations were generated by standard site-directed mutagenesis (QuikChange; Stratagene). The list of mutants is shown in Table 1 and Fig. 1(a). Full-length sequences for all constructs were verified by DNA sequence analysis.

**Transient expression of WT and mutated FcγRIIA in COS-7 cells.** Transfection of COS-7 cells with WT or mutated FcγRIIA cDNA was carried out with Lipofectamine LTX (Invitrogen),
according to the manufacturer’s protocol. Cells were examined for surface expression of FcγRIIA by flow cytometry and standard immunoblot analysis at 48 h after transfection.

**Flow cytometry.** COS-7 transfectants were washed with PBS (Invitrogen) and stained with phycoerythrin (PE)-conjugated mAb to human FcγRIIA (CD32A mAb, clone 190723; R&D Systems), according to the manufacturer’s instructions. Stained cells were analysed using a Guava EasyCyte Mini cytometer (Millipore). More than 5000 cells were counted and the results were analysed using FlowJo Version 7.5 software (Tree Star). For determining DENV infection, cells were fixed and permeabilized with 1:1 acetone:methanol mixture for 10 min and reacted with mAb 4G2 at 37 °C for 60 min. Cells were then stained with Alexa Fluor 488-labelled goat anti-mouse IgG (Invitrogen) and examined by flow cytometry.

**Electrophoresis and immunoblotting.** Cells were treated in Laemmli’s sample buffer and separated under reducing conditions by 12.5 % SDS-PAGE (Atto Corp.). Proteins were transferred to PVDF membranes (Millipore), blocked for 1 h in 5 % Immunoblock (Dainippon Sumitomo Pharma) in PBS with 0.01 % Tween 20 (PBST) and probed with anti-FcγRIIA (goat anti-human FcγRIIA/CD32a antibody; R&D Systems) at a 1:500 dilution. After washing with PBST, the blots were probed with horseradish peroxidase-conjugated anti-goat secondary antibodies (R&D Systems) at a 1:2000 dilution. Detection was performed by chemiluminescence (GE Healthcare).

**Infection of WT- or mutant FcγRIIA-transfected COS-7 cells with DENV alone or DENV–antibody complex.** DENV–antibody complex was prepared by mixing 25 μl DENV-1 or DENV-2 at titres

Fig. 5. ADE of DENV infection against COS-7 cells expressing WT and mutated FcγRIIA. (a) Immunofluorescence staining of COS-7 cells expressing WT and mutated FcγRIIA after infection with DENV–antibody complex. DENV–antibody complex was prepared by incubation of DENV-2 and anti-DENV human serum diluted 1 : 10 000. COS-7 cells were infected with the DENV-2–antibody complex and stained with mAb 4G2. The immunofluorescence photomicrographs show DENV-2 antigen-positive cells (green). The m.o.i. was 0.1. Bar, 20 μm. (b, c) The percentage of DENV-1-infected (b) or DENV-2-infected (c) cells was quantified by flow cytometry. Results are the means ± SD of three experiments performed in triplicate. The m.o.i. was 0.1. Filled bars show the mean percentage of infected cells in the presence of enhancing antibody at a 1:1000 dilution (b) or a 1:10 000 dilution (c), and open bars indicate the mean percentage of infected cells in the absence of enhancing antibody. *P < 0.05.
The mixture was prepared by mixing 25 μl of the dengue patient’s serum at a 1:1000 dilution for DENV-1 and 1:10,000 dilution for DENV-2, respectively. DENV mixtures were incubated at 37°C for 60 min with occasional agitation. For the infection assay with DENV alone, virus mixture was prepared by mixing 25 μl DENV-1 or DENV-2 at titres of 4 × 10^5 p.f.u. ml⁻¹ with 25 μl EMEM and incubated at 37°C for 60 min. COS-7 transfectants (1 × 10⁵ cells per well) were washed twice with 0.5 ml PBS and maintained in 0.5 ml EMEM supplemented with 10% FCS. DENV antigen-positive cells were determined by an immunofluorescence assay and flow cytometry at 72 h after infection.

To induce cross-linking of FcRIIA, the cells were washed with 0.1 ml PBS twice, the cells were mounted in 50% glycerol and examined under a fluorescence microscope (Olympus). Images were taken and analysed by QCapture Pro version 5.1 (QImaging). Distinct large conglomerates formed in a polar fashion at the cell margins (Kindzelskii et al., 1994; Kwiatkowska et al., 2003) were scored as cap positive, patching was scored as cross-linking, and other results were considered negative.

**Phagocytosis assay.** Opsonized *E. coli* was prepared by mixing 20 μl anti-*E. coli* rabbit pAb IgG (*E. coli* BioParticles opsonizing reagent; Invitrogen) with 6 × 10^6 SE-labelled *E. coli* BioParticles (pHrodo *E. coli* BioParticles; Invitrogen) in 1 ml EMEM supplemented with 10% FCS at 37°C for 60 min and cooled on ice for 10 min. Fifty microlitres of opsonized *E. coli* BioParticles mixture (2.5 × 10^6 SE-labelled *E. coli* BioParticles) was added to 1 × 10⁵ COS-7 cells and incubated on ice for 15 min. The cells were then incubated at 37°C for 45 min. After washing twice, the cells were analysed by flow cytometry and fluorescence microscopy.

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**REFERENCES**


![Graph showing linear regression between ADE and phagocytic activities](Image)

**Fig. 6.** Linear regression between ADE and phagocytic activities of COS-7 cells expressing WT and mutated FcRIIA. The percentage of phagocytic and DENV-infected COS-7 cells transfected with FcRIIA was reduced with the introduction of deletions and point mutations in the FcRIIA cytoplasmic domain. A significant correlation between phagocytic and ADE capacities within the COS-7 cells transfected with FcRIIA was observed with DENV-1 and DENV-2.


