An intact signal peptide on Dengue virus E protein enhances immunogenicity for CD8$^+$ T cells and antibody when expressed from Modified Vaccinia Ankara

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ABSTRACT

Dengue is a global public health concern and this is aggravated by a lack of vaccines or antiviral therapies. Despite the well-known role of $CD8^+$ T cells in the immunopathogenesis of Dengue virus (DENV), only recent studies have highlighted the importance of this arm of the immune response in protection against the disease. Thus, the majority of DENV vaccine candidates are designed to achieve protective titers of neutralizing antibodies, with less regard for cellular responses. Here, we used a mouse model to investigate $CD8^+$ T cell and humoral responses to a set of potential DENV vaccines based on recombinant modified vaccinia virus Ankara (rMVA). To enable this study, we identified two $CD8^+$ T cell epitopes in the DENV-3 E protein in C57BL/6 mice. Using these we found that all the rMVA vaccines elicited DENV-specific $CD8^+$ T cells that were cytotoxic *in vivo* and polyfunctional *in vitro*. Moreover, vaccines expressing the E protein with an intact signal peptide sequence elicited more DENV-specific $CD8^+$ T cells than those expressing E proteins in the cytoplasm. Significantly, it was these same ER-targeted E protein vaccines that elicited antibody responses. Our results support the further development of rMVA vaccines expressing DENV E proteins and add to the tools available for dengue vaccine development.

KEYWORDS

dengue virus; MVA; recombinant MVA; $CD8^+$ T cells; cytotoxic T cells; CTL.

ABBREVIATIONS

DENV, dengue virus; MVA, modified vaccinia virus Ankara; CEF, chicken embryo fibroblasts; VACV, vaccinia virus; ICS, intracellular cytokine staining.

INTRODUCTION

It is estimated that 3.6 billion people living in 124 tropical and subtropical countries are at risk of dengue virus (DENV) infections [1, 2]. Recent evaluations suggested that more than 390 million individuals are infected every year and, of these, 96 million seek medical attention [3]. DENV belongs to the *Flaviviridae* family and has four distinct serotypes: DENV-1, DENV-2, DENV-3, and DENV-4. Infection with one serotype provides life-long protection against re-infection by the same serotype, but only a short-term protection against the 3 heterologous types. The DENV E glycoprotein is a surface protein and has functions that include cell receptor binding and fusion with host cell membranes during penetration. Importantly, E is an immunodominant protein that harbors many antigenic determinants known to elicit protective immune responses [4, 5].

At present effective vaccines against dengue are not available. Several candidate vaccines have been developed and are currently being evaluated in preclinical and clinical trials [6, 7, 8]. One of the leading candidates is the Sanofi Pasteur CYD vaccine, a tetravalent live chimeric vaccine based on the 17D attenuated yellow fever virus backbone. However, despite excellent safety and immunogenicity profiles, the efficacy of this vaccine in phase 2b tests did not meet the expected threshold [9, 10]. In light of this, it is prudent to maintain a diverse pipeline of DENV vaccine candidates. Modified Vaccinia Ankara (MVA) has been shown to be a highly immunogenic recombinant vaccine vector in a number of settings, inducing good antigen-specific antibody and $CD8^+$ T cell responses [11, 12, 13, 14, 15]. Further, previous work has suggested that mucosal delivery of MVA-based vaccines can circumvent the problem of pre-existing immunity to the vector [16].

Here we present a set of MVA-vectored dengue vaccines and establish a model in C57BL/6 mice to explore their immunogenicity both for $CD8^+$ T cells and antibodies.

MATERIALS AND METHODS

Viruses and cells

Primary chicken embryo fibroblasts (CEF) were prepared as described [17] and grown in Dulbecco's Modified Eagle medium (DMEM) supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS). BHK-21 cells were grown in Minimal Essential medium (MEM) supplemented with 2 mM L-glutamine and 10% FBS. Vaccinia virus (VACV) strain MVA, and DENV-3 were gifts from Bernard Moss (NIH, Bethesda) and John Aaskov (IHBI, Brisbane), respectively.

Recombinant viruses

Recombinant MVAs (rMVA/E, rMVA/EM, rMVA/Sg-E and rMVA/Sg-EM) expressing a C-terminally truncated DENV-3 E protein were generated by homologous recombination in CEF cells (Supplementary Fig. 1). The rMVA/E and rMVA/EM contain the DNA sequence for 80% of the E glycoprotein gene (polyprotein amino acids 281 – 675), and the rMVA/Sg-E and rMVA/Sg-EM contain the same E sequence preceded by the predicted N-terminal E signal peptide (polyprotein amino acids 266 – 675). The relevant sequences were amplified by RT-PCR from a DENV-3 genotype III clinical isolate (GenBank accession # FJ850094) using the following primers: AAACCCGGGACCATGGTGGTTATTTTTATACTACTAATGCTGGTCACCCCATCC, AAACCCGGGACCATGAGATGTGTGGGAGTAGGAAACAGAGATTTTGTGGAAGG (forward primers) and TTTCTGCAGACAAAAAACATCTTCCCTTAGGAGCTTCCTTTCTTATACCAGTTG (reverse primer). Viruses rMVA/EM and rMVA/Sg-EM also have an internally located Kozak sequence

eliminated through use of the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Primers CGGAGGGTGTGTGACTTCGATGGCTAAGAACAAGC and GCTTGTTCTTAGCCATCGAAGTCACACACCCTCCG were used for mutagenesis. The PCR products were inserted into the *Sma*I and *Pst*I sites of pLW44 plasmid (provided by Bernard Moss) [18] to create transfer plasmids. Plasmid pLW44 contains homologous sequences flanking the Deletion III region of MVA genome and green fluorescent protein (GFP) under the p11 late promoter. Exogenous gene expression is controlled by the strong mH5 promoter, a late/early promoter. Transfer plasmids were sequenced and used to generate the recombinant MVAs. To this end, plasmids were transfected into CEF cells previously infected with MVA and, after 2 days, the recombinant MVAs were picked. Selection of recombinants was ensured through six successive rounds of plaque purification using the visual aid of GFP expression. MVAs were grown in BHK-21 and purified by centrifugation through a 36% sucrose cushion before titration in BHK-21 cells. Expression of the various E protein variants in infected cells was tested by western-blotting (Supplementary Fig.1C). Cells were infected at MOI of 1 and after 24 hours lysates in electrophoresis loading buffer (240 mM Tris-HCl pH 6,8; 40% glycerol; 0,8% SDS; 0,02% bromophenol blue; β-mercaptoethanol 200 mM) were obtained. Proteins were electrophoresed and blotted onto PVDF membranes (GE Healthcare, Hybond^{TM}-P). Proteins were probed using mouse anti-DENV 1+2+3+4 (ab9202, Abcam®) and rabbit anti-mouse IgG HRP (A9044, SIGMA) as secondary antibody. Blots were revealed using ECL^{TM} Plus Western Blotting Detection System (GE Healthcare) and Amersham Hyperfilm ECL (GE Healthcare).

Synthetic peptides

Putative CD8$^+$ T cell epitopes for DENV-3 E protein binding to H-2^d and H-2^b MHC I molecules were predicted as described in results. Control peptides used were: MVA $A3_{270}$,

KSYNYMLL [19] and herpes simplex virus gB_{452}, YQPLLSNTL [20]. Synthetic peptides were purchased from Mimotopes (Clayton, Australia), stocks at 10 mg/ml in dimethylsulfoxide (DMSO) were diluted to the desired concentration in serum-free DMEM.

Mice and infections

Female BALB/c, C57BL/6, and C57BL/6.SJL mice, older than 8 weeks, were obtained from the ANU Bioscience Resource Facility. For cellular analysis, mice were immunized intraperitoneally (i.p.) with 1×10^6 PFU of recombinant MVA in 200 µl of PBS. Alternatively mice were infected i.p. with 200 µl of DENV-3 (D94.283) [21]. To analyze humoral responses, mice were immunized intradermally (i.d.) [22] using two doses, 28 days apart, with each containing 10^7 PFU of MVA or 10 µg recombinant E protein plus adjuvant (saponin, Sigma) in 10 µl of PBS. All animal procedures were conducted in compliance with ethical requirements and approved by the Australian National University (ANU) Animal Ethics and Experimentation Committee.

Peptide stimulation and intracellular cytokine staining (ICS) of IFN- γ and TNF-α

Mice were euthanized 7 or 42 days after immunization and spleens were taken for analysis of $CD8^+$ T cell responses by ICS [23]. Briefly, 1×10^6 splenocytes were incubated with peptides (10^{-5} to 10^{-12} M final) at 37°C with 5% CO_2. After 1h, 1 µg of Brefeldin A (Sigma) was added to each well and plates were incubated for another 3h. Cells were then stained for surface CD8 (clone 53-6.7, BioLegend) before fixing and stained for intracellular cytokines (anti-IFN-γ-APC, clone XMG1.2; anti-TNF-α-PE-Cy7 clone MP6-XT22) in the presence of 0.5% saponin (Sigma). Results were acquired using a FACS LSR II (BD Biosciences) and analyzed with Flowjo software (Tree Star Inc.). Backgrounds were determined by using control wells with no peptides for each mouse and were subtracted from the values of test samples.

Staining of CD107a/b

Splenocytes were incubated with peptides as above but with the addition of Golgi-Stop (BD Biosciences) and anti-CD107a/b-FITC. After this incubation, cells were stained for surface CD8 and intracellular IFN-γ. Acquisition, analysis and subtraction of background were as described above.

In vivo cytotoxicity assay

Splenocytes from C57BL/6.SJL ($CD45.1^+$) mice were pulsed 10^{-6} M peptide for use as targets. Three treatments of these were done with different peptides and amounts of CFSE label: 1) gB_{452}, as an irrelevant peptide and 5 µM CFSE; 2) $D3E_{284-292}$ and 0.5 µM CFSE; 3) $D3E_{408-415}$ and no CFSE. These cells were mixed in equal proportion and a total of 5×10^6 injected by intravenous (i.v.) injection into C57BL/6 mice 7 days after immunization. Mice were euthanized after 22 h and the recovery of each of the cell populations described above determined by flow cytometry. The following formula was used to determine specific lysis. Ratio = (percentage $CFSE^{high}$/ percentage $CFSE^{low}$) for $D3E_{284-292}$ or ratio = (percentage $CFSE^{high}$/ percentage $CFSE^{neg}$) for $D3E_{408-415}$. Percent specific lysis = [1 – (ratio naïve mouse / ratio infected mouse) x 100].

ELISA

Serum was taken 14 days after the final immunization and tested in duplicate for anti-E IgG antibodies. Nunc Maxisorp® plates were coated overnight with recombinant DENV-3 E protein (100 ng/well) in bicarbonate buffer. After blocking for 2 hours with 5% skim milk in PBS-T, diluted serum samples (1/100) were added to the plates and incubated for 1 hour at room temperature. Bound antibody was detected with anti-mouse IgG-HRP (1:10000) (#7076, Cell Signaling) and TMB substrate at 100 µl/well. Absorbance at 450 nm was then measured.

Statistical analysis

Comparisons were made using one-way ANOVA followed by post analysis with Bonferroni's multiple comparison tests (GraphPad Prism v5.00). Differences were considered significant when $p \leq 0.05$.

RESULTS

Identification of $CD8^+$ T cell epitopes in DENV-3 E

To enable comparison of DENV-3 E-specific $CD8^+$ T cell responses in a mouse model, we first needed to identify H-2-restricted epitopes in this protein. Some progress has been made in identifying DENV-specific $CD8^+$ T cell epitopes, but most are in non-structural proteins and restricted to human MHC alleles [24, 25, 26]. Epitope identification work in mice has focused largely on DENV-2 [27, 28]. For the DENV-3 E protein, several $CD8^+$ T cell epitopes have been predicted, but only one has been shown to be immunogenic in DENV infection [29, 30, 31]. We predicted H-2^d- and H-2^b-binding peptides from the DENV-3 E protein sequence (FJ850094) combining results from BIMAS, IEDB and SYFPEITHI databases [32, 33, 34]. From these predictions, thirteen peptides were synthesized, in each case we used only the longest version of any predicted binding sequence irrespective of rank (Table 1).

Next, the peptides were used to restimulate splenocytes taken from mice 7 days after immunization with rMVA/E (Supplementary Fig. 1) and their ability to induce IFN-γ production by $CD8^+$ T cells detected by ICS. In C57BL/6 mice, $D3E_{284\text{-}292}$ (VGVGNRDFV) and $D3E_{408\text{-}419}$ (KVVQYENLKYTV), stimulated IFN-γ production by $CD8^+$ T cells (Table 1 and Figure 1A) from mice immunized with rMVA/E, but not MVA. These initial screens used 10^{-5} M peptide so to validate and refine our mapping they were tested at concentrations down to 10^{-12} M.

184 $D3E_{284-292}$ was able to restimulate a half-maximal response at between 10^{-9} and 10^{-10} M and
185 we concluded that this sequence was likely to be optimal (Figure 1B). However, half-maximal
186 responses to $D3E_{408-419}$ were $>10^{-8}$ M (Figure 1C). To map a minimal H-2^b-restricted epitope
187 in this region, we tested a range of peptide variations at a wide range of concentrations
188 (Figure 1D). $D3E_{408-415}$ (KVVQYENL) had a half maximal response at the lowest
189 concentration among all peptides and was chosen as the most likely minimal epitope. None of
190 the predicted H-2^d-restricted peptides were active in assays using splenocytes from
191 immunized BALB/c mice and no further work was done in this strain.

192 **$D3E_{284-292}$ and $D3E_{408-415}$ are recognized by $CD8^+$ T cells during DENV-3 infection**

193 To ensure the epitopes mapped using our vaccine were also presented during DENV
194 infection, $D3E_{284-292}$ and $D3E_{408-415}$ were tested for their ability to stimulate IFN-γ production
195 by $CD8^+$ T cells from spleens of C57BL/6 mice infected 7 days earlier with a passage 1 stock
196 of DENV-3 [21]. Figure 1E and 1F shows that $D3E_{284-292}$ and $D3E_{408-415}$ were both epitopes in
197 the context of a DENV-3-infection. Of these, $D3E_{284-292}$ (VGVGNRDFV) has been previously
198 predicted [30], but not shown to be elicited by DENV infection and the more dominant $D3E_{408-415}$ is entirely novel.

200 **ER-targeted E protein is more immunogenic for $CD8^+$ T cells**

201 We then used the epitopes to investigate whether there were differences in immunogenicity
202 across a set of rMVA vaccines. These vaccines, namely rMVA/E, rMVA/EM, rMVA/Sg-E and
203 rMVA/Sg-EM, all express 80% of DENV-3 E, but rMVA/Sg-E and rMVA/Sg-EM address the
204 protein to the endoplasmic reticulum (ER) via the native signal sequence. In addition,
205 rMVA/EM and rMVA/Sg-EM had a potential internal Kozak sequence eliminated by
206 mutagenesis, in case this compromised translation of full length E protein. Western blotting
207 showed that the vaccines expressed similar levels of E protein, irrespective of the design

(Supplementary Fig. 1). Groups of mice were immunized with each of the recombinant viruses and $CD8^+$ T cell responses to the DENV-3 and several native MVA epitopes were measured at the peak of the acute response (7 days) and in memory (42 days). On day 7, there were significantly more $CD8^+$ T cells responding to the DENV peptides when measured by detecting IFN-γ alone, IFN-γ and CD107, or IFN-γ and TNF-α in mice immunized with MVA vectors expressing ER-targeted E protein, compared to those without the signal sequence (Figure 2). However, there was no apparent advantage in mutating the internal Kozak sequence (Figure 2).

For memory responses, the trend for higher responses elicited by vaccines with E proteins having signal sequences was maintained, but only reached statistical significance for $D3E_{284-292}$ when measured by co-expression of intracellular IFN-γ and surface CD107 (Figure 3). In all experiments responses to native MVA epitopes were similar across the vaccines (not shown).

DENV-3 vaccines induce cytotoxic $CD8^+$ T cells

To ensure that our vaccines elicited DENV-3-specific cytotoxic $CD8^+$ T cells, we assessed the ability of immunized mice to eliminate peptide loaded targets *in vivo* (Figure 4) [23, 35]. For this experiment we immunized mice with rMVA/Sg-E, rMVA/E or used MVA as a control and the ability of mice to kill cells loaded with $D3E_{284-292}$ and $D3E_{408-415}$ was tested 7 days later. As expected, control MVA-immunized mice were unable to kill either of the D3E-peptide loaded targets. Mice immunized with each of the two rMVA DENV-3 vaccines were able to kill targets loaded with either $D3E_{284-292}$ or $D3E_{408-415}$. There was no significant difference in killing between the vaccines, but the mean specific lysis for rMVA/Sg-E was higher for both epitopes.

ER-targeted E protein generates superior antibody responses

Given the established role for antibodies in protection against DENV, it was important to determine if changing the cellular location of the E protein also altered humoral responses. To test this we immunized mice with rMVA/E, rMVA/Sg-E or controls, giving two doses of 10^7 PFU 28 days apart and then measuring antibodies by ELISA two weeks after the boost. Whereas rMVA/Sg-E was able to induce an easily detectable antibody response, rMVA/E elicited no response distinguishable from background (Figure 5).

DISCUSSION

The quest for an effective dengue vaccine has recently reached a milestone with a phase 2b clinical trial of the Sanofi Pasteur CYD vaccine in Thailand. However, the overall efficacy was only 30.2% suggesting a requirement for alternative strategies [9, 10].

Almost all neutralizing antibodies are directed against epitopes in the DENV E protein and for this reason it is an obvious choice for dengue subunit vaccines. $CD8^+$ T cell epitopes in E are likely to be less immunodominant than others in non-structural proteins [25, 28, 29], but the renewed interest in cellular immunity suggests these should not be ignored [25, 28, 36]. With this in mind we wanted to evaluate $CD8^+$ T cell immunity to the E protein in our rMVA vectored dengue vaccines prior to testing antibody responses, but this required first identifying $CD8^+$ T cell epitopes for DENV-3 E. Variants of $D3E_{284\text{-}292}$ in other flaviviruses have previously been shown to be $CD8^+$ T cell epitopes with some cross-reactivity across these viruses [30]. We now show that this is also an epitope of DENV-3 and responses to this peptide are also elicited by rMVA E protein vaccines. $D3E_{408\text{-}415}$ is entirely novel and it is also significantly more dominant than $D3E_{284\text{-}292}$. Indeed in mice immunized with rMVA vaccines, $D3E_{408\text{-}415}$ was more immunogenic than the native MVA $A3_{270\text{-}277}$ epitope used for comparison, which is second ranked in the MVA immunodominance hierarchy [37]. In contrast

to C57BL/6 mice, none of the predicted H-2^d-binding peptides were found to be immunogenic in the context of our MVA vaccine. This may reflect the reliability of the predictive algorithms for H-2^d or the relatively poor immunogenicity of MVA in BALB/c mice [38].

Our evaluation of rMVA/E vaccines demonstrates the value of $CD8^+$ T cell epitope information in vaccine development. Here we show that a modification of the DENV-3 E protein that strongly improves humoral responses is also most likely superior for eliciting $CD8^+$ T cells. The improvement in $CD8^+$ T cell responses were statistically significant at acute times and in every memory experiment the mean response was higher for groups immunized with the ER-targeted E protein, even if generally not reaching significance. We speculate that this may also be the case for E proteins expressed from vectors other than MVA.

Recent studies for several infectious diseases, including dengue, suggest that the quality of $CD8^+$ T cells, as demonstrated by their ability to exert multiple functions, is important [39, 40, 41, 42, 43, 44, 45]. For example, higher frequencies of $CD8^+$ T cells expressing TNF-α, IFN-γ, and IL-2 in Thai children were associated with the development of subclinical, rather than clinical secondary infection [46]. Another study showed that higher and more polyfunctional responses in the context of particular HLA alleles were associated with a decreased susceptibility to severe disease [25]. Together these suggest that a vigorous response by multifunctional $CD8^+$ T cells is important for protection against dengue. All rMVA vaccines evaluated here elicited responses that included co-incident production of IFN-γ and TNF-α or production of IFN-γ and degranulation . In general the ability of $CD8^+$ T cells to produce cytokines after stimulation is in the order: IFN-γ, TNF-α and IL-2 [47]. In this context, the fraction of IFN-γ^+ $CD8^+$ T cells also making TNF-α was approximately 80% for all the vaccines, suggesting that all elicit a response of similar quality (not shown). Finally, all vaccines induced a response that was able to kill targets displaying DENV-3 E-derived

peptides *in vivo*. In summary, these results suggest that the $CD8^+$ T cell response elicited by the rMVA vaccines tested here is polyfunctional and importantly this was not reduced by modifying the E protein to improve antibody responses.

In conclusion, we provide new tools for pre-clinical analysis of cellular immune responses to DENV vaccines and use them to demonstrate that the requisites for a good antigen can coincide for humoral and $CD8^+$ T cell immunity. This work adds to previous studies of an MVA vector expressing DENV proteins tested in non-human primates [48]. Our results support further development of MVA vectored dengue vaccines and show that targeting DENV E to the ER improves cellular and humoral immune responses to this important antigen.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicting interests.

ACKNOWLEDGMENTS

We thank Prof John Aaskov and the W.H.O. Arbovirus Reference Centre at the Queensland University of Technology for provision of DENV-3 isolates. We also thank Dr Ricardo Gazzinelli for continuous support. Parts of this work were supported by the *Instituto Nacional de Ciência e Tecnologia de Vacinas* – INCTV (National Institute of Science and Technology of Vaccines) and by a FAPEMIG PPM grant (CBB - PPM-00461-11). BRQ was a CAPES/PDSE fellowship recipient (8815-11-9). FGF is a CNPq fellowship recipient. DCT is an ARC Future Fellow (FT110100310).

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449 **FIGURE LEGENDS**

450

451 **Figure 1. Mapping of $CD8^+$ T cell epitopes in DENV-3 E glycoprotein.** Mice were
452 immunized i.p. with MVA, rMVA/E or DENV-3 isolate D94.283 (approx. 10^3 PFU) and 7 days
453 later, percentages of $CD8^+$ T cells producing IFN-γ in response to the peptides shown were
454 measured by ICS. (A) Representative flow cytometry plots for mice immunized with rMVA/E or
455 MVA. Nil, control with no peptide; $A3_{270}$, native epitope of MVA. (B) Fraction of $CD8^+$ T cells
456 responding to $D3E_{284-292}$ (VGVGNRDFV) at the indicated concentrations. (C) As for B, but
457 using $D3E_{408-419}$ (KVVQYENLKYTV). (D) Redefining the $D3E_{408-419}$ peptide. Fraction of $CD8^+$
458 T cells responding to the variants of $D3E_{408-419}$ as shown. (E) Representative flow cytometry
459 plots for mice immunized with DENV-3, rMVA/Gg-E or MVA. Peptides as for panel A. (F) The
460 percent of $CD8^+$ T cells from each immunization group producing IFN-γ after stimulation with
461 the peptides shown. Data are the mean and SEM from a group of four DENV-3-infected mice
462 and are individual mice for the other immunizations. Results in panel F were independently
463 reproduced using a second isolate of DENV-3. For other panels, data are representative of 2
464 (panels C and D) or 3 (panel B) experiments.

465

466 **Figure 2. Immunogenicity of rMVA/DENV-3E vaccines at acute times.** C57BL/6 mice were
467 immunized i.p. with 10^6 PFU of rMVA/Sg-E, rMVA/Sg-EM, rMVA/E or rMVA/EM and 7 days
468 later, splenocytes were used in ICS assays. (A and B) Percentages of $CD8^+$ splenocytes that
469 produced IFN-γ after stimulation with $D3E_{284-292}$ and $D3E_{408-415}$ peptides, respectively. Data
470 are represented as means and SEM from 3 experiments (C and D) Percentages of
471 $CD8^+/CD107ab^+$ splenocytes that produced IFN-γ after stimulation with $D3E_{284-292}$ and
472 $D3E_{408-415}$ peptides, respectively. Data are represented as means and SEM from 2

473 experiments. (E and F) Percentages of $CD8^+$/IFN-γ^+ splenocytes that produced TNF-α after
474 stimulation with the peptides $D3E_{284-292}$ and $D3E_{408-415}$, respectively. Data are represented as
475 means and SEM from 2 experiments. Peptide concentration was 10^{-7} M. *, $p \leq 0,5$; **, $p \leq$
476 0,01; ***, $p \leq 0,001$.

477

478 **Figure 3. Immunogenicity of rMVA/DENV-3E vaccines in memory.** C57BL/6 mice were
479 immunized i.p. with 10^6 PFU of rMVA/Sg-E, rMVA/Sg-EM, rMVA/E or rMVA/EM and 42 days
480 later, splenocytes were used in ICS assays. (A and B) Percentages of $CD8^+$ splenocytes that
481 produced IFN-γ after stimulation with $D3E_{284-292}$ and $D3E_{408-415}$ peptides, respectively. (C and
482 D) Percentages of $CD8^+$/$CD107ab^+$ splenocytes that produced IFN-γ after stimulation with
483 $D3E_{284-292}$ and $D3E_{408-415}$ peptides, respectively. (E and F) Percentages of $CD8^+$/IFN-γ^+
484 splenocytes that produced TNF-α after stimulation with the peptides $D3E_{284-292}$ and $D3E_{408-415}$, respectively. Peptide concentration was 10^{-7} M. Data are represented as means and SEM
486 from 3 experiments. *, $p \leq 0,5$; **, $p \leq 0,01$; ***, $p \leq 0,001$.

487

488 **Figure 4. Cytotoxic capacity of DENV-3E-specific $CD8^+$ T cells.** A) Specific lysis of
489 $D3E_{284-292}$ peptide loaded cells. B) Specific lysis of $D3E_{408-415}$ peptide loaded cells. Data are
490 represented as means and SEM from 2 experiments. *, $p \leq 0,5$; **, $p \leq 0,01$; ***, $p \leq 0,001$.

491

492 **Figure 5. Humoral immunogenicity for rMVA/DENV-3E vaccines.** C57BL/6 mice were
493 immunized i.d. through a homologous prime-boost protocol with 10^7 PFU of rMVA/Sg-E or
494 rMVA/E and, 14 days later, serum samples were used in ELISA assays to detect anti-E IgG

antibodies. Data are represented as means and SEM from 2 experiments. *, $p \leq 0,5$; **, $p \leq 0,01$; ***, $p \leq 0,001$.

Supplementary Figure 1. Generation of recombinant MVAs expressing truncated DENV-3 E glycoproteins. (A) To generate the recombinants MVA (rMVA/Sg-E, rMVA/Sg-EM, rMVA/E and rMVA/EM), transfer plasmids were used to drive the homologous recombination. These transfer plasmids were constructed by cloning the different cDNAs for E glycoprotein in the pLW44 plasmid, resulting in plasmids pLW44/Sg-E, pLW44/Sg-EM, pLW44/E, pLW44/EM. Plasmids pLW44/Sg-E and pLW44/Sg-EM contain 80% E protein and its preceding signal sequence (Sg), whereas in pLW44/Sg-EM an undesired internal Kozak sequence was eliminated by mutation (M). Plasmids pLW44/E and pLW44/EM only contain 80% E protein. Plasmid pLW44/EM also had the internal Kozak sequence mutated. (B) Representative scheme highlighting the Kozak sequences. Kozak sequences (in grey) are present in the start ATG (bold and underlined) and in a downstream ATG codon (only underlined). Plasmids pLW44/Sg-EM and pLW44/EM had this downstream Kozak sequence eliminated by mutation. Mutated bases are in bold and italic. (C) Western Blot analysis of C-terminally truncated DENV-3 E protein expression in MVA-infected (1 PFU/cell) or control BHK21 cells as indicated above each lane. For each sample, lysates containing 30 µg of total protein extracts were separated on 12% polyacrylamide gels and western blots were probed with mouse anti-DENV 1+2+3+4 antibodies. Positive control (+ve) is DENV-3 E protein produced by *E. coli*.

TABLE

Table 1. DENV-3E epitopes predicted.

Name[a]	**Sequence**	**MHC**	**% IFN-γ$^+$ (of CD8$^+$)**[b]
D3E$_{284-292}$	VGVGNRDFV	H-2D^b	0.27
D3E$_{313-321}$	TMAKNKPTL	H-2D^b	-
D3E$_{365-375}$	EQDQNYVCKHT	H-2D^b	-
D3E$_{369-377}$	NYVCKHTYV	H-2D^b/H-2K^d	-
D3E$_{408-419}$	KVVQYENLKYTV	H-2D^b/H-2K^b	1.17
D3E$_{411-420}$	QYENLKYTVI	H-2K^d/H-2D^b	-
D3E$_{416-423}$	KYTVIITV	H-2K^d/H-2D^b	-
D3E$_{429-439}$	HQVGNETQGVT	H-2D^b	-
D3E$_{443-453}$	TPQASTTEAIL	H-2L^d	-
D3E$_{463-471}$	CSPRTGLDF	H-2D^d/H-2L^d	-
D3E$_{489-496}$	QWFFDLPL	H-2D^b/H-2K^d	-
D3E$_{540-548}$	TALTGATEI	H-2D^b/H-2K^d	-
D3E$_{576-586}$	SYAMCTNTFVL	H-2K^d	-

[a] Numbers in subscripts represent the amino acid positions in DENV-3 polyprotein (GenBank accession nº FJ850094).

[b] Data are presented as means values from groups of 3 mice.

FIG.1

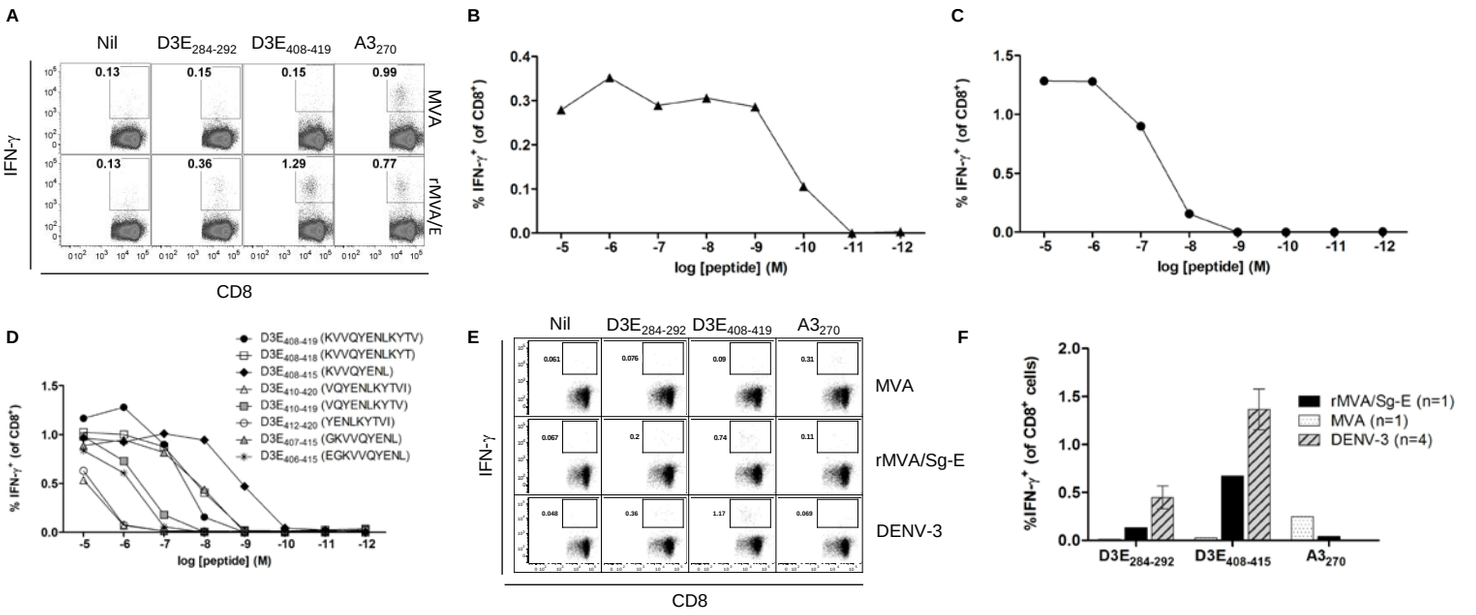

A
Nil
D3E284-292
D3E408-419
A3270
0.13
0.15
0.15
0.99
0.13
0.36
1.29
0.77
MVA
rMVA/E
IFN-γ
CD8
B
% IFN-γ+ (of CD8+)
log [peptide] (M)
C
% IFN-γ+ (of CD8+)
log [peptide] (M)
D
D3E408-419 (KVVQYENLKYTV)
D3E408-418 (KVVQYENLKYT)
D3E408-415 (KVVQYENL)
D3E410-420 (VQYENLKYTVI)
D3E410-419 (VQYENLKYTV)
D3E412-420 (YENLKYTVI)
D3E407-415 (GKVVQYENL)
D3E406-415 (EGKVVQYENL)
% IFN-γ+ (of CD8+)
log [peptide] (M)
E
Nil
D3E284-292
D3E408-419
A3270
0.061
0.076
0.09
0.31
0.057
0.2
0.74
0.11
0.048
0.36
1.17
0.069
MVA
rMVA/Sg-E
DENV-3
IFN-γ
CD8
F
rMVA/Sg-E (n=1)
MVA (n=1)
DENV-3 (n=4)
%IFN-γ+ (of CD8+ cells)
D3E284-292
D3E408-415
A3270

FIG.2

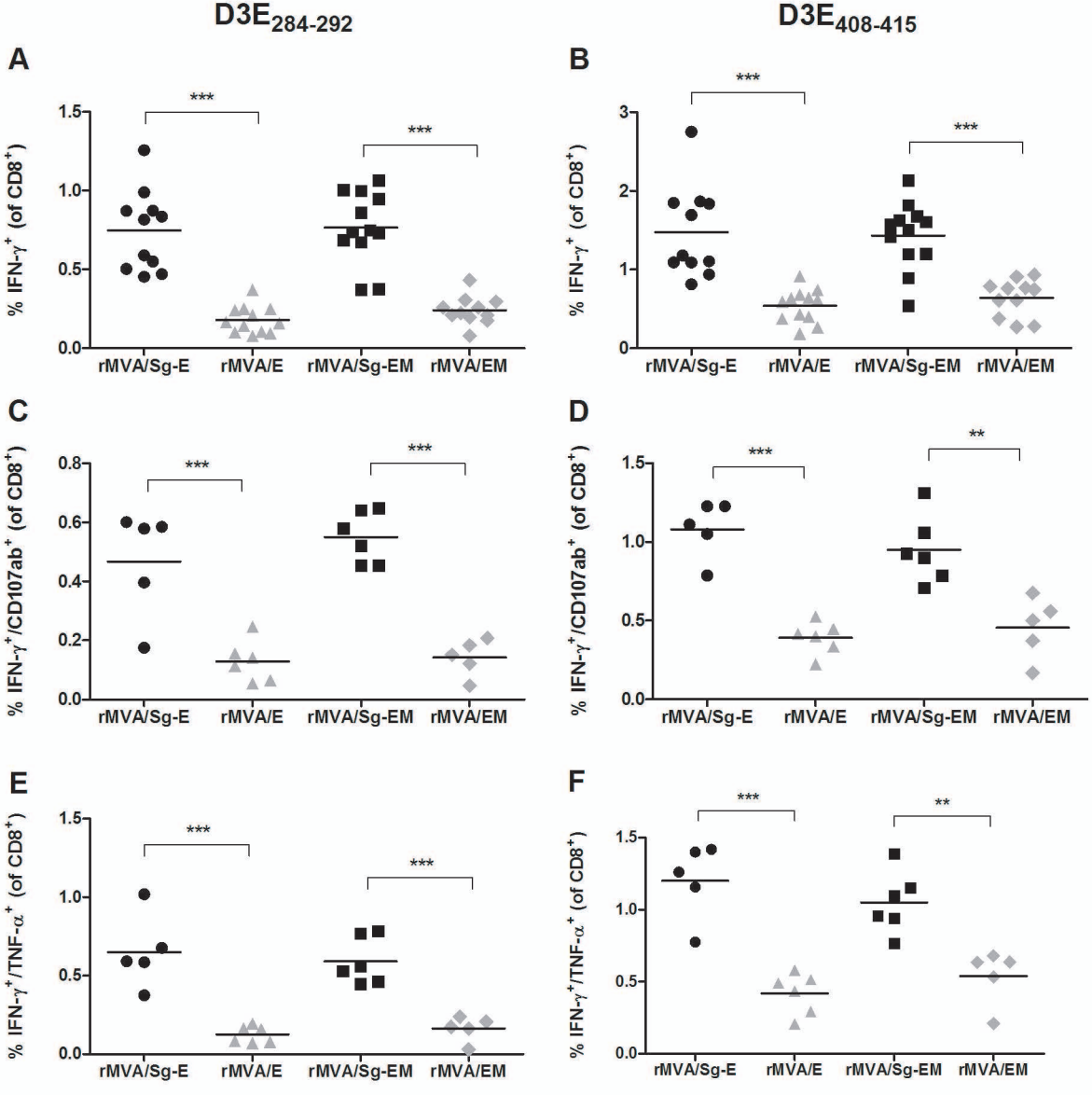

D3E284-292
D3E408-415
A
B
C
D
E
F
% IFN-γ+ (of CD8+)
% IFN-γ+/CD107ab+ (of CD8+)
% IFN-γ+/TNF-α+ (of CD8+)
rMVA/Sg-E
rMVA/E
rMVA/Sg-EM
rMVA/EM
***
**

FIG. 3

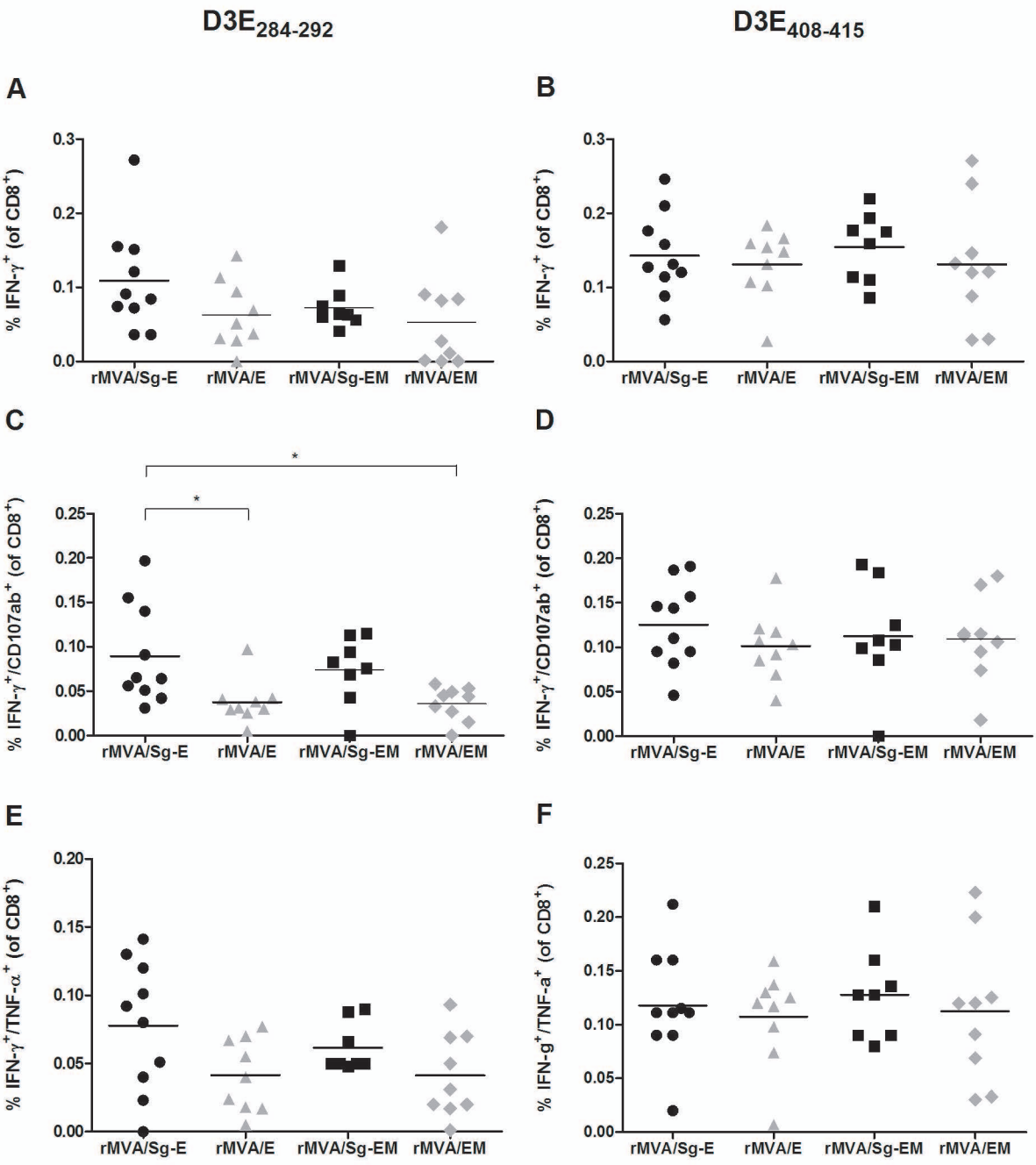
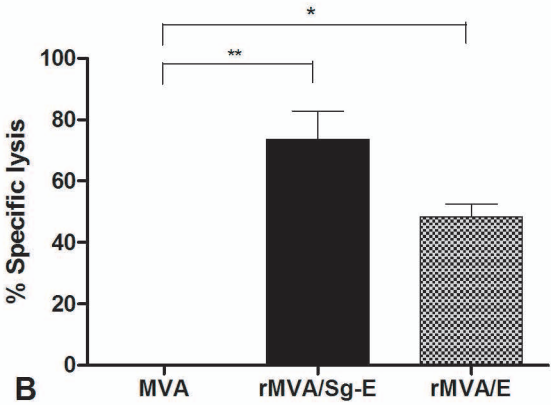

D3E284-292
D3E408-415
A
B
C
D
E
F
% IFN-γ+ (of CD8+)
% IFN-γ+/CD107ab+ (of CD8+)
% IFN-γ+/TNF-α+ (of CD8+)
% IFN-g+/TNF-a+ (of CD8+)
0.0
0.1
0.2
0.3
0.05
0.15
0.25
rMVA/Sg-E
rMVA/E
rMVA/Sg-EM
rMVA/EM
*
*

FIG.4

A


*
**
100
80
60
40
20
0
% Specific lysis
MVA
rMVA/Sg-E
rMVA/E

B

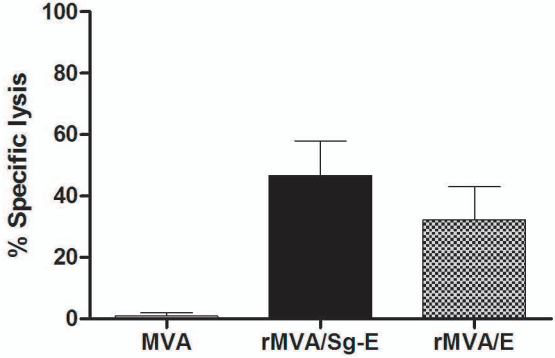
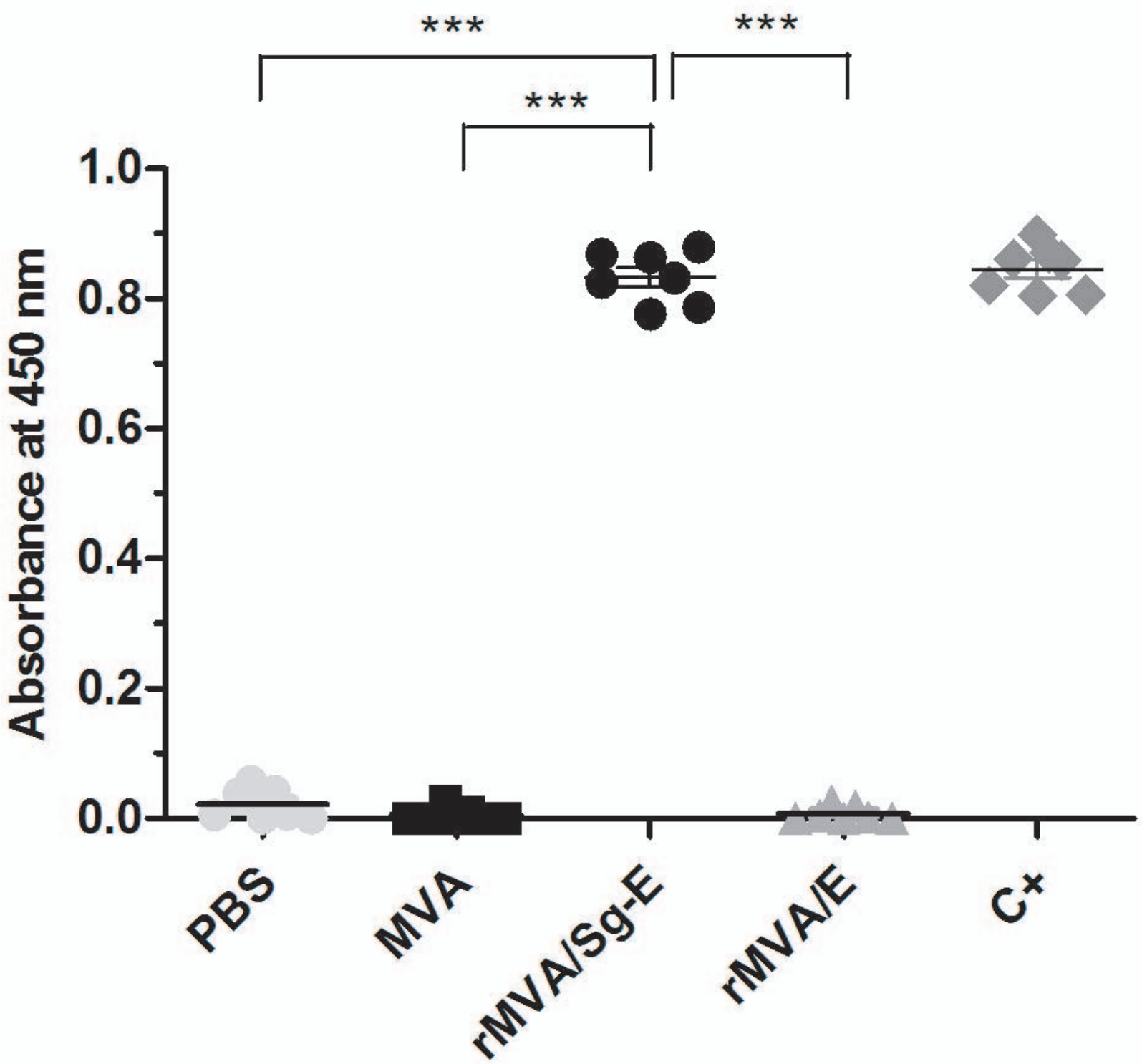
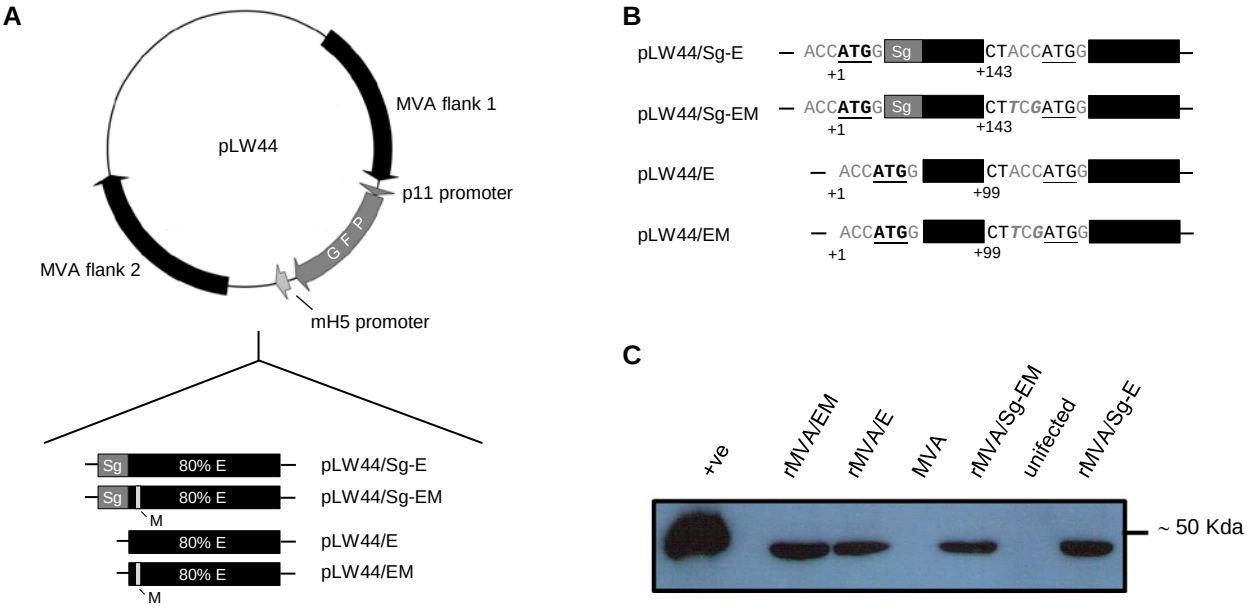

100
80
60
40
20
0
% Specific lysis
MVA
rMVA/Sg-E
rMVA/E

FIG.5



SUPP FIG.1


A
pLW44
MVA flank 1
p11 promoter
G F P
MVA flank 2
mH5 promoter
Sg
80% E
pLW44/Sg-E
pLW44/Sg-EM
M
pLW44/E
pLW44/EM
B
pLW44/Sg-E
pLW44/Sg-EM
pLW44/E
pLW44/EM
+1
+143
+99
C
+ve
rMVA/EM
rMVA/E
MVA
rMVA/Sg-EM
unifected
rMVA/Sg-E
~ 50 Kda