Recombinant glycoprotein B vaccine formulation with Toll-like receptor 9 agonist and immune-stimulating complex induces specific immunity against multiple strains of cytomegalovirus

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INTRODUCTION

Human cytomegalovirus (CMV) is a betaherpesvirus that can cause primary infection through transmission via multiple routes such as saliva, sexual contact, breastfeeding, placental transfer, blood transfusion, solid organ transplantation and haematopoietic stem cell transplantation, and then establishes latency with periodic reactivation in the host (Crough & Khanna, 2009; Gandhi & Khanna, 2004). In immunocompetent individuals, primary infection is mostly asymptomatic; however, it can lead to symptomatic illness such as infectious mononucleosis and splenomegaly in some individuals. Due to the disease burden of primary CMV and reinfection during pregnancy, development of a vaccine to prevent congenital CMV was given the highest priority by the US Institute of Medicine in its 1999 assessment of targets for vaccine development (Arvin et al., 2004). The potential target population for vaccination would be women of child-bearing age and transplant recipients prior to immunosuppressive treatment. Recently, a subunit vaccine based on CMV glycoprotein B (gB) formulated with MF59 adjuvant successfully concluded phase II clinical trials (Pass et al., 2009). The gB/MF59 vaccine showed 50% efficacy in young mothers, and congenital CMV infection occurred in 1/81 subjects (1%) in the vaccinated group and 3/97 subjects (3%) in the placebo group (Pass et al., 2009). The
primary objective of this vaccine was to elicit neutralizing antibodies in vaccinated individuals similar to natural CMV infection (Pass, 2009), and the results from this study clearly indicated that this vaccine provided some protection similar to natural CMV infection. There is an emerging argument that, to improve the efficacy of the CMV gB vaccine, it will be essential to induce a humoral and cellular immune response. Indeed, previous studies by Klein et al. (1999) showed that human sera from naturally infected individuals often fail to neutralize heterologous CMV isolates. Furthermore, previous studies carried out in pregnant women and transplant recipients have also suggested the importance of virus-neutralizing antibodies in limiting virus dissemination and reinfection with heterologous strains (Boppana et al., 2001; Ishibashi et al., 2007; Ross et al., 2010). In addition, the loss of T-cell immunity in advanced human immunodeficiency virus/AIDS and transplant recipients significantly compromises the ability of the host to restrict viral infection and replication (Barry et al., 2007; Harari et al., 2004; Reusser et al., 1991). Based on these observations, we explored the capacity of different formulations of the CMV gB vaccine to induce humoral and cellular immune responses and to control multiple strains of CMV expressing different gB genotypes. Here, we have shown that a formulation of CMV gB vaccine including a Toll-like receptor 9 (TLR9) agonist and immune-stimulating complexes (ISCOMs) is highly effective in inducing strong antiviral humoral and cellular immune responses. These responses were capable of neutralizing multiple strains of CMV with differing gB genotypes, and of providing protection against challenge with recombinant vaccinia virus expressing gB.

RESULTS

Evaluation of the immunogenicity of recombinant gB in combination with a TLR9 agonist and/or ISCOMs

To evaluate the best compatible adjuvant combination for the CMV gB vaccine, groups of human leukocyte antigen (HLA)-A2/Kb transgenic mice (five mice per group) were immunized with 50 μg recombinant gB protein in combination with 50 μg per dose of CpG ODN1826 (TLR9 agonist) and/or 13 μg per dose of AbISCO 100 (ISCOM) intramuscularly. In addition, animals immunized with adjuvants or gB alone were used as a negative control, whilst mice immunized intramuscularly with an adenovirus vector encoding CMV gB (Ad-gB, 7.5 × 10⁸ p.f.u. per mouse) were used as a positive control (Fig. 1a). These animals were sacrificed at 10 days post-immunization and assessed for CMV gB-specific T-cell immunity using an intracellular cytokine staining assay, and humoral responses were assessed using a gB ELISA. All mice vaccinated with CMV gB protein formulated with AbISCO 100, with or without CpG ODN1826, showed high levels of antibody titres comparable to those generated with the Ad-gB vaccine (Fig. 1b). Low or undetectable gB-specific antibody responses were observed in mice immunized with gB alone or adjuvants (AbISCO 100 and CpG ODN1826) alone. In contrast, the assessment of gB-specific T-cell responses using a gamma interferon (IFN-γ) intracellular cytokine staining assay revealed that gB vaccine formulated with both AbISCO 100 and CpG ODN1826 was the only combination to induce significant cellular immunity. All other combinations induced low or undetectable T-cell responses (Fig. 1c). The T-cell responses induced by the gB vaccine formulated with both AbISCO 100 and CpG ODN1826 were significantly higher than those of the Ad-gB vaccine. Taken together, these analyses suggested that a gB vaccine formulation with AbISCO 100 and a TLR9 agonist can elicit strong humoral and cellular responses.

Optimization of CMV gB vaccine dose for vaccination

Having established that a CMV gB vaccine could potentially be formulated to induce both humoral and cellular immune responses, we next conducted a series of experiments to identify the optimal dose of gB protein for this vaccine formulation. HLA-A2/Kb transgenic mice were immunized with varying doses of gB protein (5, 10, 50 and 100 μg) in combination with AbISCO 100 and CpG ODN1826. Ten days after vaccination, the animals were assessed for CMV-specific antibody and T-cell responses (Fig. 2a). These analyses showed that 5 μg gB protein in combination with AbISCO 100 and CpG ODN1826 was sufficient to induce an optimal antibody response (Fig. 2b) and CD4⁺ T-cell response (Fig. 2c). To explore the generation of CD8⁺ cytotoxic T-lymphocyte responses, mice were assessed for their reactivity to previously mapped T-cell epitopes from the gB protein (WQGIKQKSLVEVLARNSS, SMESVHLNLYAQLQFTYDTL and GRCSYRVPFNPANSYVQ). Optimal CD8⁺ T-cell responses were also generated with 5 μg gB (Fig. 2c). The level of antibody and T-cell responses were comparable to those generated in mice immunized with 50 or 100 μg gB. No detectable gB-specific immune response was observed in control mice immunized with AbISCO 100 and CpG ODN1826 alone.

Prime–boost immunization with CMV gB vaccine formulated with AbISCO 100 and CpG ODN1826 dramatically improves immune responses

To evaluate this novel CMV gB vaccine formulation further, we tested the immunogenicity of this vaccine using a prime–boost strategy. HLA-A2/Kb mice were immunized with gB formulated with AbISCO 100 and CpG ODN1826 and then boosted with the same formulation on day 21 (Fig. 3a). Animals immunized with either a prime or boost dose alone, or with a prime–boost of adjuvant alone, were included as controls for this analysis. CMV gB-specific humoral and cellular immune responses
were initially assessed 10 days after the boost injection (30 days after the initial priming). The data presented in Fig. 3(b–d) show that prime–boost vaccination increased both gB-specific antibody and CD4⁺ T-cell responses, although no significant differences were evident in the CD8⁺ T-cell response. Mice were sacrificed 54 days after the booster immunization (i.e. 75 days after the initial priming) to assess the impact of prime–boost immunization on the long-term humoral and cellular memory responses. Although the overall antibody and T-cell responses were lower on day 75 compared with day 30, these responses were significantly higher than those observed in mice given prime immunization alone (Fig. 3e–g). To confirm further the T-cell responses detected following prime–boost immunization, splenocytes from these mice were stimulated with gB protein and then assessed for IFN-γ production using intracellular cytokine staining assays. The data presented in Fig. 3(h) show strong expansion of gB-specific CD8⁺ T cells, as shown by the increase in IFN-γ in the presence of gB-specific peptides (difference range 0.31–2.84 %) from animals immunized with the CMV gB vaccine using a prime–boost strategy. Taken together, these observations clearly demonstrated that prime–boost immunization with recombinant CMV gB protein in combination with AbISCO 100 and CpG ODN1826 generated both humoral and cellular responses against gB that could be sustained in the long term.
Prime–boost immunization with the CMV gB vaccine induces high-avidity antibody responses that neutralize multiple strains of CMV

Generation of a high-avidity antibody response against the gB protein is crucial for the successful control of CMV infection in vivo. In the next set of experiments, we assessed the avidity of the gB-specific antibody response induced in mice immunized with the CMV gB vaccine. The data presented in Fig. 4(a) show that, although prime–boost immunization increased the antibody titre compared with prime immunization alone, it did not alter the gB-specific antibody avidity. Next, we assessed whether this high-avidity antibody response was capable of neutralizing CMV strains expressing different genotypes of gB protein (i.e. gB1, gB2, gB3 and gB4), as previous observations have suggested that neutralizing antibodies are not necessarily cross-reactive to multiple CMV strains following natural infection (Klein et al., 1999). Antiviral neutralizing activity in serum samples from animals receiving prime alone or prime–boost immunizations were assessed using micro-neutralizing assays against the four different strains of CMV. The data presented in Fig. 4(b) show that serum samples from prime–boost animals displayed higher anti-CMV neutralizing titres when compared with the prime alone or placebo groups. Furthermore, the neutralizing titres in prime–boost animals were comparable to those seen in serum samples from healthy virus carriers (Fig. 4b). More importantly, the antibody response generated in the prime–boost group showed a strong neutralizing capacity against multiple strains of CMV expressing different genotypes of gB. The data from these experiments clearly showed that antibodies from mice immunized with the prime–boost strategy induced a high-avidity antibody response that was capable of neutralizing multiple genotypes of CMV.

Immunization with CMV gB in combination with AbISCO 100 and CpG ODN1826 induces a polyfunctional CMV-specific T-cell response

A number of recent studies have implicated a role for T-cell polyfunctionality, including the capacity to generate...
Fig. 3. Short- and long-term CMV-specific memory humoral and cellular immune responses following immunization with CMV gB vaccine using a prime–boost strategy. (a) Multiple groups of HLA-A2/Kb mice were immunized intramuscularly with 5 μg CMV gB protein formulated with AbISCO 100 and CpG ODN1826 or with adjuvants alone. A booster dose was given on day 21 to mice allocated to the prime–boost strategy and then sacrificed at 10 days (day 30) or 54 days (day 75) after the final immunization. (b, e) Evaluation of CMV gB-specific antibody titres using ELISA. (c, d, f, g) Assessment of CMV gB-specific CD4+ and CD8+ T-cell responses following immunization. Splenocytes from the mice were stimulated with gB protein or peptide epitopes and then assessed for IFN-γ production using intracellular cytokine staining assays. The data represent means ± SEM from five mice in each group. (h) Splenocytes from the prime–boost group (75 days post-immunization) were stimulated in vitro with gB peptide epitopes as outlined in Methods and cultured for 10 days in the presence of recombinant IL-2. These cells were assessed for gB-specific T-cell reactivity using intracellular cytokine staining assays. The data in the dot plots represent the percentage of IFN-γ-producing CD8+ T cells from five individual mice (m1–m5).
multiple cytokines [IL-2, IFN-\(\gamma\) and tumour necrosis factor alpha (TNF-\(\alpha\)], in protection against CMV infection (Almeida et al., 2007; Harari et al., 2007; Precopio et al., 2007). To determine whether the CMV gB vaccine formulation induced a polyfunctional T-cell response, CMV-specific CD4\(^+\) T cells from vaccinated mice were analysed for their ability to produce IFN-\(\gamma\), TNF-\(\alpha\) and IL-2 following ex vivo stimulation with gB protein. Splenocytes from these mice were isolated on days 30 (effector phase) and 75 (memory phase), stimulated with gB protein and the frequency of CD4\(^+\) T cells producing multiple cytokines was analysed by fluorescence-activated cell sorting (FACS) and calculated by Boolean gating analysis. Analysis of the three cytokines allowed identification of seven distinct populations of gB-specific CD4\(^+\) T cells and the proportions of CD4\(^+\) T cells expressing various combinations of cytokines. The data from this analysis are presented in Fig. 5. This analysis revealed that the majority of the gB-specific effector and memory CD4\(^+\) T cells were triple (IFN-\(\gamma^+\), TNF\(^+\) and IL-2\(^+\)) or double (IFN-\(\gamma^+\), TNF\(^+\) and IL-2\(^-\)) cytokine producers. The proportion of triple cytokine producers increased as the CD4\(^+\) T cells moved from the effector phase to the memory phase of the immune response, indicating that the CMV gB vaccine formulation is highly effective at inducing and maintaining a polyfunctional virus-specific T-cell response. Another interesting feature of these virus-specific T cells was that a large proportion of these cells produced IL-2, which suggests that these cells may support the expansion of CD8\(^+\) T cells following secondary antigen encounter.

**Immunization with CMV gB in combination with AbISCO 100 and CpG ODN1826 affords protection against quasi-viral infection**

Having established the immunogenicity of the CMV gB vaccine formulation, our next experiment was designed to determine the protective efficacy of this vaccine. Due to the species restriction of CMV, we challenged immunized HLA-A2/K\(^b\) mice with recombinant vaccinia virus encoding the gB protein (Vacc-gB; Ad169 CMV strain) to evaluate the protective efficiency of the CMV gB vaccine (Fig. 6a). The data presented in Fig. 6(b) show that HLA-A2/K\(^b\) mice immunized with CMV gB vaccine showed a significant reduction in virus load following challenge with Vacc-gB when compared with the control mice that were mock immunized with adjuvants alone. A two- to fourfold reduction in viral load was observed in animals immunized with the CMV gB vaccine.

**DISCUSSION**

The data presented in this study clearly show that a CMV gB vaccine formulation that combines a TLR9 agonist and ISCOMs can be used successfully to generate strong cross-neutralizing humoral and cellular immune responses. We also showed that a prime–boost vaccination strategy can dramatically enhance the effector and long-term memory T-cell responses. It is important to stress that these immune responses were induced over a wide range of gB concentrations (5–100 \(\mu\)g) and are well within the range of concentrations used for human trials (Pass et al., 2009). One of the interesting features of the cellular immune response is the ability of these responses to expand and maintain their function over time, which is crucial for long-term protection against CMV infection.
Novel CMV gB vaccine with TLR9 agonist and ISCOMs

Fig. 5. Cytokine expression by CMV-specific CD4+ T cells from CMV gB-vaccinated mice. Ex vivo expression of IFN-γ, TNF-α, IL-2 and IL-2 by antigen-specific CD4+ T cells from mice immunized with the CMV gB vaccine formulation. Splenocytes were prepared from HLA-A2/Kb mice at 30 (a) and 75 days (b) post-vaccination and cultured with gB protein. Brefeldin A was added during the last 6 h of incubation, which was followed by T-cell surface marker and intracellular cytokine staining. The proportions of CD4+ T cells producing multiple cytokines on days 30 and 75 were split into seven different populations based on Boolean gating analysis of IFN-γ, TNF and IL-2. Each of the different populations expressing distinct sets of cytokines is indicated with a different colour.

response induced by this novel CMV gB vaccine was the polyfunctionality of antigen-specific T cells. A large proportion of T cells generated following vaccination were triple (IFN-γ+, TNF+ and IL-2+) or double (IFN-γ+, TNF+ and IL-2+) cytokine producers. More importantly, this polyfunctional profile was sustained long term after vaccination. These cytokines play a vital role in protection and in the development of a memory immune response. TNF plays a crucial role in the establishment and maintenance of the microarchitecture in secondary lymphoid organs (Kuprash et al., 1999) and protection against infectious pathogens (Flynn et al., 1995; Suresh et al., 2004). Of particular interest was the expression of IL-2 by the majority of these effector cells, which indicates that these cells have a high proliferative capacity and also provide help in the generation and maintenance of antiviral CD8+ T-cell responses (Cousens et al., 1995; Smith, 1988; Willerford et al., 1995).

CMV displays a broad host-cell range and is able to infect various cell types such as endothelial cells, epithelial cells, smooth muscle cells, fibroblasts, leukocytes and dendritic cells (Hahn et al., 2004; Percivalle et al., 1993; Riegler et al., 2000; Sinzger et al., 1995). Infection of endothelial cells by CMV has been regarded as a possible virulence factor that might influence the clinical course of infection (Gerna et al., 2002; Sinzger et al., 1999). Recent studies have shown that epithelial entry-specific neutralizing antibody titres from gB/MF59-vaccinated individuals were several fold lower when compared with CMV natural infection (Cui et al., 2008). Another major challenge in designing an effective CMV vaccine is that the immune response induced following vaccination should be able to prevent reinfection with heterologous strains of CMV. Indeed, recent studies have shown that maternal reinfection by new strains of cytomegalovirus is a major source of congenital infection in a highly CMV-immune population (Boppana et al., 2001; Mussi-Pinhata et al., 2009; Ross et al., 2010). On the basis of sequence variation in the UL55 gene encoding the gB protein, CMV can be classified into four major gB genotypes: gB1, gB2, gB3 and gB4. A number of studies have suggested that genetic variation of CMV strains may correlate with their pathogenicity in CMV-associated diseases (Nogueira et al., 2009; Roubalová et al., 2010). Although there is no direct evidence, it has been proposed that antibody responses directed towards one particular genotype may not be effective against other genotypes and thus will be ineffective in preventing reinfection with heterologous strains of CMV (Boppana et al., 1999, 2001; Urban et al., 1992). It is important to stress here that our gB vaccine formulation did not target other potential neutralizing components of CMV (e.g. UL128–131) that are crucial for entry of the virus in endothelial cells, epithelial cells and dendritic cells (Hahn et al., 2004; Percivalle et al., 1993; Riegler et al., 2000; Sinzger et al., 1995), but we were successful in inducing a robust cellular immunity (CD8+ and CD4+ T cells) that may overcome this potential limitation and thus improve the protective efficacy of the CMV gB vaccine. Indeed, recent studies by Hansen et al. (2010) showed that antiviral CD8+ T-cell responses play a crucial role in preventing superinfection of rhesus CMV-infected rhesus macaques.

Taken together, the data presented in this study showed that a CMV gB vaccine formulated with AbiISCO 100 and CpG ODN1826 induces a humoral immune response with cross-neutralizing activity against heterologous strains of CMV expressing different genotypes of gB. Furthermore, the immune response induced by this vaccine was also effective in providing strong resistance against viral infection, as demonstrated by the challenge with recombinant vaccinia virus expressing the gB protein. These observations are supported by earlier studies by Wang et al. (2004) who also showed that prior immunization with modified vaccinia virus Ankara
encoding the gB protein can induce neutralizing antibodies against CMV strains of different genotypes. Future studies should be designed to compare various gB vaccine formulations (including recombinant gB protein with MF59 and alum) to determine the most efficient formulation for the prevention of CMV infection. Most importantly, the data presented in this study provide an insight for the design of protein-based vaccines against viral infections aiming to induce both humoral and cellular immune responses and also overcome the potential limitations of the use of viral vector-based vaccines with respect to their safety concerns in the healthy human population.

**METHODS**

**Generation of the CMV gB expression construct and protein purification.** An expression vector encoding the soluble form of gB protein from CMV strain Ad169 (with the transmembrane region of the sequence deleted and mutations in the furin cleavage site of Arg433→Gln, Arg435→Thr and Arg436→Gln) with a tissue plasminogen signal sequence was cloned into the pCEP4 vector (Invitrogen). The resulting plasmid, pCEP4gB, was purified using a Qiagen Maxiprep kit and transiently transfected into Freestyle HEK293 human embryonal kidney cells with 293fectin reagent (Invitrogen). Protein expression was carried out in Freestyle expression medium (Invitrogen) at 37°C and 5% CO₂ in Erlenmeyer flasks for 48–72 h and the protein was purified by affinity chromatography using a gB-specific mAb (Singh & Compton, 2000).

**Generation of recombinant adenovirus encoding CMV gB protein.** The DNA sequence encoding gB was amplified from the AD169 virus stock by PCR using gene-specific primers. This PCR product was designed to encode the gB sequence from Ala31 to Val700 with deletion of the signal sequence. Following amplification, the DNA was cloned into a pBluescript II K+S phagemid and confirmed by DNA sequence analysis. Assembly and production of the recombinant adenovirus encoding gB was carried out as described previously (Zhong et al., 2008).

**Animal immunization.** HLA-A2 transgenic mice (Animal Resource Centre, Canning Vale, WA, Australia) containing human HLA-A*0201 with murine α3 chain (referred to as HLA-A2/Kb) (Engelhard et al., 1991; Newberg et al., 1992) were maintained in a pathogen-free animal facility at the Queensland Institute of Medical Research (QIMR). All protocols were followed in compliance with the QIMR Animal Ethics Committee. Six- to 8-week-old female mice were immunized intramuscularly with varied concentrations of gB protein and AbISCO 100 adjuvant (Isonova) and/or CpG ODN1826 (InvivoGen) followed by determination of the CMV-specific humoral and cellular immune response at various time points. In addition, mice immunized intramuscularly with Ad-gB (7.5 × 10⁸ p.f.u. per mouse) were used as a positive control.

**ELISA assay to determine gB antibody titres of vaccinated mice.** Anti-gB antibody titres were determined using ELISA as described previously (Zhong et al., 2004). Briefly, polystyrene 96-well half-area plates (Costar; Corning) were coated with 1 µg recombinant CMV gB protein ml⁻¹ and incubated at 4°C overnight. Serially diluted sera from vaccinated mice, control (placebo) mice or pooled serum from healthy seropositive individuals were added to the wells
and incubated for 2 h at room temperature. Twenty-five microlitres of HRP-conjugated goat anti-mouse Ig (H+L) antibody (Southern Biotech) was added to each well and incubated for 1 h at room temperature, followed by the addition of 25 μl per well of the substrate 3,3',5,5'-tetramethylbenzidine (eBioscience). The reaction was stopped by adding 25 μl 1 M HCl and the absorbance at 450 nm was determined. Results were expressed as titres or reciprocal titres.

Assessment of gB-specific antibody avidity. CMV gB-specific avidity was evaluated as described previously (Marshall & Adler, 2003). In brief, serial dilutions of sera from immunized animals were prepared in duplicate and, to induce dissociation, 5 M urea in PBS (PBS containing 0.05% Tween 20), or PBS alone as a control, was added to the appropriate wells and then incubated with HRP-conjugated goat anti-mouse Ig for 1 h. The plates were then developed as outlined for the ELISA.

Microneutralization assay against heterologous strains of CMV. Neutralizing activity was determined against four different strains of CMV: Towne (gB1 type), AD169 (gB2 type), Toledo (gB3 type) and 57A (gB4 type). The assay procedure was followed as described previously (Wang et al., 2004). In brief, human fibroblast MRC-5 cells were plated in 96-well flat-bottomed plates. The next day, complement-inactivated serum samples from gB-vaccinated mice, control mice (placebo) or pooled sera from healthy seropositive individuals were serially diluted and added to a standard number of virus particles (1000 p.f.u. per well) diluted in 30 μl R0 (RPMI with no serum) in 96-well U-bottomed plates and incubated for 2 h at 37 °C and 5% CO2. As a positive control, virus without serum and a negative-control serum without virus were also included in the test. The serum/CMV mixture was then added to the MRC5 cells and incubated at 37 °C and 5% CO2 for 2 h. After incubation, the mixture was discarded and the cells washed gently five times with RPMI containing 10% FCS (R10) and a final volume of 200 μl R10 was added to each well, followed by incubation for 16–18 h at 37 °C and 5% CO2. After incubation, the cells were fixed with 100 μl chilled methanol and incubated with Peroxidase Block (Dako), followed by mouse anti-CMV IE-1/IE2 mAb (Chemicon) for 3 h at room temperature. Cells were then incubated with 50 μl HRP-conjugated goat anti-mouse Ig (diluted 1:200 in PBS) per well for 3 h at room temperature. In the final step, the cells were stained with 20 μl diaminobenzidine plus substrate (Dako) per well for 10 min at room temperature and positive nuclei that stained dark brown were counted. The neutralizing titre was calculated as the reciprocal of the serum dilution that gave 50% inhibition of IE-1/IE-2-expressing nuclei.

T-cell responses assessed by intracellular cytokine staining. To determine the gB CD4+ T-cell response, approximately 1 × 106 splenocytes (50 μl per well) in 96-well V-bottomed plates were stimulated with 2.5 μg gB or Dulbecco's modified Eagle's medium (DMEM) (as a negative control).

CD8+ T-cell responses were assessed by stimulating 50 μl splenocytes with gB peptides WQGIKQSKLVELERLARSS, MESVHNLVYA-QLOQFTYDTL and GRCSYRPVIFENFANSYYVQ (2 μg ml⁻¹). These peptides were identified from previous experiments in which we stimulated gB-vaccinated mice splenocytes with a gB matrix (different mixtures of 20mer peptides overlapping by 10 aa) that, together, covered the full-length of the gB protein and should contain all possible T-cell epitopes. After incubation with these peptides for 2 h at 37 °C and 5% CO2, 150 μl per well of DMEM containing 0.3 μl of Brefeldin A (BD Pharmingen) was added and the plates were incubated overnight. Cells were then stained with allophycocyanin (APC)-conjugated anti-CD8, FITC-conjugated anti-CD4 and peridinin-chlorophyll protein complex-conjugated anti-CD8 (all from BD Pharmingen) mAbs resuspended in PBS containing 2% FCS and incubated at 4 °C for 30 min. Cells were fixed with Cytofix (BD Pharmingen) and permeabilized by washing twice with 1 × Cytoperm (BD Pharmingen). Cells were then incubated with PE-conjugated anti-IFN-γ mAb (BD Pharmingen) and APC-conjugated anti-IL-2 (BD Pharmingen) for multiple cytokine analysis, and analysed using a BD FACSCanto.

Statistical analysis. Statistical analyses were carried out using Microsoft Office Excel 2007 and GraphPad Prism 4 software. For antibody titres, neutralizing antibody titres and cellular immune responses, the means ± SD were calculated and P values were determined by Student's t-test. Error bars represent SEM.

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