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(72) Inventors; and

(71) Applicants : **YAN, Huimin** [CN/CN]; No.1 Teaching Building, No.44 Middle District of Xiaohongshan, wuhan, Hubei 430071 (CN). **LI, Moming** [CN/CN]; No1 Teaching Building, NO.44 Middle District of Xiaohongshan, wuhan, Hubei 430071 (CN). **ZHOU, Dihan** [CN/CN]; No.1 Teaching Building, No.44 Middle District of Xiaohongshan, Wuhan, Hubei 430071 (CN). **YU, Jie** [CN/CN]; No.1 Teaching Building, No.44 Middle District of Xiaohongshan, Wuhan, Hubei 430071 (CN).

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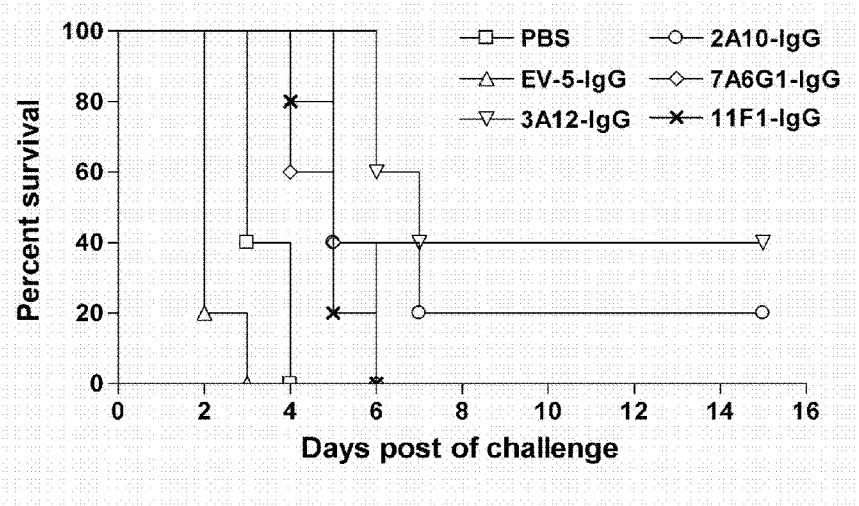


FIG 4

(57) Abstract: A monoclonal immunoglobulin G (IgG) antibody specifically binding to a peptide, wherein the peptide is represented by a consensus sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2. An immunogenic composition comprises a recombinant 3D protein or a 3D protein derived immunogenic polypeptide, and a pharmaceutically acceptable adjuvant.

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# IMMUNOGLOBULIN G MONOCLONAL ANTIBODIES AGAINST 3D PROTEINS OF ENTEROVIRUSES

## Field of the Invention

**[001]** The present invention generally relates to prophylactic and therapeutic agents against enteroviruses, and more particularly to monoclonal immunoglobulin G (IgG) antibodies (mAbs) specific for 3D proteins of enteroviruses, and further to immunogenic compositions comprising 3D proteins or the peptide with the epitopes recognized by the monoclonal IgG antibodies against 3D proteins.

## Background of the Invention

**[002]** Enteroviruses are grouped into four major subtypes, A, B, C and D, and each subtype contains many serotypes. Enteroviruses cause diverse diseases. For instance, the major causative agents of hand, foot, and mouth disease (HFMD) are enterovirus 71 (EV71) and coxsackievirus (CV) that belong to the family *Picornaviridae*. HFMD represents a growing threat to public health, especially for young children. The infection with EV71 and CV causes severe aseptic meningitis, encephalitis, myocarditis, acute faccid paralysis, and pulmonary edema, resulting in high fatality rates.

**[003]** As a member of the genus *Enterovirus* within the family *Picornaviridae*, EV71 has a typical positive-sense single stranded RNA genome with a single open reading frame encoding four capsid proteins (VP1-4) and seven nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D). 3D (also called $3D^{pol}$) protein acts as a viral RNA-dependent RNA polymerase (RdRp) and plays a major

role in viral negative-strand synthesis and the uridylylation of a few proteins. From the studies of the crystal structure of EV71, one EV71 virion contains an outer capsid formed by 60 copies of each of three viral structural proteins VP1-VP3 and the inner surface of the capsid is attached by 60 copies of a small protein VP4.

**[004]** 3D has high sequence identity in all enteroviruses, but has a low homology with human proteins. The EV71 3D generally shares structure/sequence similarity with homologous RdRps from poliovirus, coxsackievirus, rhinovirus and foot-and-mouth disease virus polymerases in *Picornaviridae* family.

**[005]** 3D has an N-terminal active site. Kiener et al. used a recombinant 3CD protein from EV71 C4 strain as an immunogen and isolated a monoclonal antibody 4B12 (IgG1) recognizing a linear epitope DFEQALFS (corresponding to positions 53-60 of 3D and 1784-1791 of the EV71 polyprotein) close to the active site of the 3D polymerase; 4B12 detected all enterovirus 71 subgenotypes in a denaturing dot blot assay (Kiener et al. Characterization of a monoclonal antibody against the 3D polymerase of enterovirus 71 and its use for the detection of human enterovirus A infection. *J Virol Methods.* 2012; 180(1-2):75-83). The detection of broad subgenotype strains by 4B12 strongly suggested that 3D-specific mAb might be useful for diagnostic of infection, but no prophylactic or therapeutic use of 4B12 has ever mentioned or suggested, let alone general 3D-specific antibodies.

**[006]** Since vaccine is the best strategy to control the infectious diseases, various EV71 vaccine candidates have been investigated, including inactivated EV71 whole virus vaccines, live-attenuated virus vaccine, recombinant VP1 vaccine, VP1-based DNA vaccine, synthetic peptide vaccine and virus-like particle vaccine. Formalin-inactivated EV71 vaccines elicit satisfactory levels of immunoprotection and cross-reactivity neutralization the antibodies in mice and rhesus monkeys; in China, inactivated EV71 vaccines have completed Phase III clinical trials in 2013. Since the outer capsid contains the major antigenic sites during infection, synthetic peptide vaccines included the peptides only from capsid proteins including VP1 and

VP2 (Kung et al. Update on the development of enterovirus 71 vaccines. *Expert Opin Biol Ther.* 2014; 3:1-10).

**[007]** Because EV71 is a RNA virus, the replication of such EV71 capsid gene by the error-prone RdRp might result in considerable genetic and antigenic diversity. Chen et al. demonstrated that with a panel of monoclonal antibodies against VP1, the genotypes do not reflect their antigenicity, and EV71 viruses could be classified into different antigenic groups (Chen et al. Antigenic analysis of divergent genotypes human Enterovirus 71 viruses by a panel of neutralizing monoclonal antibodies: current genotyping of EV71 does not reflect their antigenicity. *Vaccine.* 2013;31(2):425-30). All these demonstrated that it is a challenge for choosing an ideal strain for the development of a vaccine with broad effectiveness.

**[008]** Since no vaccine or specific antiviral is currently available, human intraveneous immunoglobulin (IVIG) has been clinically used in treating severe EV71 infections. However, the discovery of antibody dependent enhancement (ADE) of EV71 infection illustrates the complex roles of antibody in controlling EV71 infection. ADE is a phenomenon in which preexisting sub-levels of neutralizing antibodies enhance virus entry and replication. Sub-levels of neutralizing antibodies was evidenced to enhance EV71 infection in Fc receptor-bearing human monocytes and contributed to exacerbation of EV71 infection in mice. Furthermore, the wide existence of cross reactivity between enterovirus antibodies may also become the underlying risk for the ADE during EV71 infections.

**[009]** Han et al. demonstrated that lower concentration (50μg/ml) of neutralizing antibodies (IVIG) against EV71 can enhance EV71 infection in a monocytic cell line (Han et al. Antibody dependent enhancement infection of enterovirus 71 *in vitro* and *in vivo*. *Virol J.* 2011; 8:106). Cao et al. further studied the roles of each IgG subclass on neutralization and enhancement of EV71 infection, and found that the neutralizing activity of human IVIG is mainly mediated by IgG1 subclass and to less extent by IgG2 subclass, and IgG3 fraction did not have neutralizing activity but enhanced EV71 infection *in vitro* (Cao et al., Human IgG

subclasses against enterovirus Type 71: neutralization versus antibody dependent enhancement of infection. *PLoS One.* 2013; 8(5):e64024). Therefore, novel vaccine should be designed to induce the production of antibodies with strong neutralizing but weaker ADE activities.

**[0010]** Since structural capsid proteins have antigenic diversity due to mutations, it is difficult to identify universal antibodies against them; so far, two universal IgG monoclonal antibodies have been identified, one against VP1 (Lim et al. Characterization of an isotype-dependent monoclonal antibody against linear neutralizing epitope effective for prophylaxis of enterovirus 71 infection. *PLoS One.* 2012; 7(1):e29751), and another against VP3 (Kiener et al. A novel universal neutralizing monoclonal antibody against enterovirus 71 that targets the highly conserved "knob" region of VP3 protein. *PLoS Negl Trop Dis.* 2014; 8(5):e2895). However, the ADE capacity of these universal antibodies was not studied.

**[0011]** Serious safety concerns for the inactivated EV71 virus vaccines have been raised by the studies of Jia et al (Jia et al. The cross-reactivity of the enterovirus 71 to human brain tissue and identification of the cross-reactivity related fragments. *Virol J.* 2010; 7:47). Jia et al. showed the presence of specific IgG in the EV71 infected patient sera, having the cross-reactivity activity to human cerebra; then using 19 purified peptides to prepare polyclonal sera, the anti-sera of P230-323, P646-755, P857-1012 and P1329-1440 showed strong staining with neuron plasma in both adult human cerebra and fetus medulla, the anti-sera of P1-69, P324-443, P444-565, P566-665, P746-876, P1441-1526, P1549-1668, P1732-1851 and P2072-2193 showed weaker staining, and the anti-sera of P70-159, P140-249, P1197-1338, P1649-1731 and P1843-1951 did not show staining with both adult human cerebra and fetus medulla sections.

**[0012]** Therefore, there is an imperative need to develop new means against enteroviruses such as EV71 and CV causing HFMD.

## Summary of the Invention

**[0013]** The present invention provides a monoclonal immunoglobulin G (IgG) antibody specifically binding to a peptide. In one embodiment, the peptide is represented by a consensus sequence selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO: 2.

**[0014]** In another embodiment of the monoclonal IgG antibody, the monoclonal IgG antibody binding to the peptide represented by SEQ ID NO: 1 is 3D-2A10-IgG (CCTCC NO: C2014143, and the monoclonal IgG antibody binding to the peptide represented by SEQ ID NO: 2 is 3D-3A12-IgG (CCTCC NO: C2014141).

**[0015]** In another embodiment of the monoclonal IgG antibody, the monoclonal IgG antibody is selected from the group consisting of an antibody comprising an Fc domain, a single-chain antibody, and a Fab fragment.

**[0016]** In another embodiment of the monoclonal IgG antibody, the peptide represented by SEQ ID NO: 1 is selected from the group consisting of the peptides represented by SEQ ID NOS: 3-8, and the peptide represented by SEQ ID NO: 2 is selected from the group consisting of the peptides represented by SEQ ID NOS: 10-14.

**[0017]** The present invention also provides an immunogenic composition. In one embodiment, the immunogenic composition comprises a recombinant 3D protein or a 3D protein derived immunogenic polypeptide, and a pharmaceutically acceptable adjuvant.

**[0018]** In another embodiment of the immunogenic composition, the recombinant 3D protein is selected from the group consisting of the 3D proteins represented by SEQ ID NOS: 15-22, and variants with at least 85% identity to corresponding wild-type 3D proteins.

**[0019]** In another embodiment of the immunogenic composition, the 3D protein derived immunogenic polypeptide comprises a first peptide represented by SEQ ID NO: 1, a second peptide represented by SEQ ID NO: 2, and an artificial linker, wherein the first and second peptide are covalently coupled by the linker. In a

further embodiment of the immunogenic composition, the first peptide is selected from the group consisting of the peptides represented by SEQ ID NOS: 3-8, and the second peptide is selected from the group consisting of the peptides represented by SEQ ID NOS: 10-14. In another further embodiment of the immunogenic composition, the artificial linker is selected from the group consisting of the linkers represented by SEQ ID NOS: 23 and 24.

**[0020]** The objectives and advantages of the invention will become apparent from the following detailed description of preferred embodiments thereof in connection with the accompanying drawings.

## Brief Description of the Drawings

**[0021]** Preferred embodiments according to the present invention will now be described with reference to the Figures, in which like reference numerals denote like elements.

**[0022]** FIG 1. Characterization of EV71 3D-specific monoclonal IgG antibodies. (A) Pictures showing indirect immunofluorescence staining of EV71-infected cells with 3D-specific IgG mAbs (3A12, 2A10, 7A6G1, and 11F1); a flagellin-specific mAb (5G10) was included as a negative control, and 3D-immunized mice serum as positive control. (B) Western blot of EV71-infected Vero-1008 cell lysates with 3D-specific IgG mAbs (3A12, 2A10, 7A6G1, and 11F1); a flagellin-specific mAb (5G10) was included as a negative control, and 3D-immunized mice serum as positive control.

**[0023]** FIG 2. 3D-specific IgG mAbs intracellularly inhibited EV71 replication. (A) A graph showing the virus titers with the presence of intracellular antibodies by transfection. (B) A graph showing the virus titers with the presence of different doses of 2A10 or EV-5. EV-5 is a MAb specific for VP2 of EV71.

**[0024]** FIG 3. 3D-specific IgG mAbs inhibited *in vitro* 3D polymerase activities. (A) A schematic diagram illustrating 3D (RdRp)-mediated RNA elongation.

(B) A picture showing the effects of different IgG mAbs on 3D-mediated RNA elongation.

**[0025]** FIG 4. A graph showing the antiviral efficacies of EV71 3D-specific IgG mAbs in an *in vivo* murine model.

**[0026]** FIG 5. A graph showing the antibody-dependent enhancement of EV71 replication in the presence of 2A10-IgG or EV-5-IgG mAbs.

**[0027]** FIG 6. Epitopes of EV71 3D-specific 3A12 and 2A10 and its space situation in three dimension model of EV71 3D (1RA6). EV71 3D (1RA6) was used to indicate position of the two identified epitopes of 3A12 (orange) and 2A10 (light teal).

**[0028]** FIG 7. Western blot of 3D expression by VTT-3D expression vector (VTT-3D, a attenuated Vaccinia virus expressing 3D).

**[0029]** FIG 8. A diagram showing the immunization and challenge protocol.

**[0030]** FIG 9. Graphs showing the 3D-specific antibody responses after prime immunization: (A) serum IgG; (B) serum IgA; (C) saliva IgA; and (D) vagina IgA.

**[0031]** FIG 10. Graphs showing the 3D-specific antibody responses after first boost: (A) serum IgG; (B) serum IgA; (C) saliva IgA; and (D) vagina IgA.

**[0032]** FIG 11. Graphs showing the 3D-specific antibody responses after second boost: (A) serum IgG; (B) serum IgA; (C) saliva IgA; and (D) vagina IgA.

**[0033]** FIG 12. A graph showing the 3D-specific IgG antibody responses in the intestine of neonatal mice after birth from the immunized mothers.

**[0034]** FIG 13. A graph showing the percent survival of the neonatal mice under a mice-adapted strain EV71 challenge.

**[0035]** FIG 14. Graphs showing antibody responses after immunization with purified 3D proteins.

## Detailed Description of the Invention

**[0036]** The present invention may be understood more readily by reference to the following detailed description of certain embodiments of the invention.

**[0037]** Throughout this application, where publications are referenced, the disclosures of these publications are hereby incorporated by reference, in their entireties, into this application in order to more fully describe the state of art to which this invention pertains.

**[0038]** The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, for example, *Molecular Cloning: A Laboratory Mannual*, second edition (Sambrook et al., 1989); *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987).

**[0039]** The present invention discovered that 3D protein of enterovirus 71 (EV71), a BrCr strain from Enterovirus A subtype as an example, was immunogenic eliciting specific immune responses and protected hosts from the challenges of EV71. Further studies generated 3D protein specific monoclonal immunoglobulin G (IgG) antibodies that could inhibit EV71 replication without inducing antibody-dependent enhancement (ADE) effect and protect host from challenges when administered into the host. In addition, the epitopes bound by the neutralizing antibodies can be linked by an artificial linker to form immunogenic polypeptides.

**[0040]** 3D protein of EV71 BrCr strain (SEQ ID NO: 15) was expressed and purified, and purified 3D protein was used to immunized mice for generating monoclonal IgG antibodies following standard protocols. Two 3D-protein specific monoclonal IgG antibodies (3D-2A10-IgG and 3D-3A12-IgG) were generated by conventional hybridoma technology. The corresponding hybridoma cell lines have been deposited at China Center for Type Culture Collections (CCTCC), CCTCC NO: C2014143 for 3D-2A10-IgG, and CCTCC NO: C2014141 for 3D-3A12-IgG. The monoclonal IgG antibody can be an antibody comprising an Fc domain, a single-chain

antibody, or a Fab fragment. The definition and production of the variety of antibodies are well known.

**[0041]** Using recombinant 3D proteins with sequential deletions from either ends, the binding peptides for antibodies were identified. 3D -3A12-IgG binds to KEPAVLTS (SEQ ID NO: 3), and 3D-2A10-IgG binds to YSTYVKDELRSLDKI (SEQ ID NO: 9). Using sequence alignment, the identified peptides are highly conserved in all enterovirus strains from enterovirus subtypes A, B, C and D. As shown in Table 1 hereinbelow, 3D-3A12-IgG binds to a peptide that is represented by a consensus sequence of KEPAVL$X_7X_8$ (SEQ ID NO: 1), where $X_7$ is selected from the group consisting of T, H, R and N, and $X_8$ from the group consisting of S, N and K. 3D-2A10-IgG binds to a peptide that is represented by a consensus sequence of $X_1X_2TX_4$VKDELRS$X_{12}X_{13}KX_{15}$ (SEQ ID NO: 2), where $X_1$ is selected from the group consisting of Y, M, L and F, $X_2$ from the group consisting of S and V, $X_4$ from the group consisting of Y and F, $X_{12}$ from the group consisting of L, A, K and R, $X_{13}$ from the group consisting of D, E, T and S, and $X_{15}$ from the group consisting of I and V.

**[0042]** Table 1. Consensus sequences and exemplary sequences recognized by 3D-2A10-IgG or 3D-3A12-IgG

| Sequence Name | Sequence | SEQ ID NO |
|---|---|---|
| **3D-3A12-IgG** | | |
| Consensus | KEPAVL$X_7X_8$ | SEQ ID NO: 1 |
| EV71 BrCr | KEPAVLTS | SEQ ID NO: 3 |
| EV71 Henan | KEPAVLHS | SEQ ID NO: 4 |
| CA9 | KEPAVLRN | SEQ ID NO: 5 |
| CA16 | KEPAVLTS | SEQ ID NO: 3 |
| CVB1 | KEPAVLRN | SEQ ID NO: 5 |
| CVB3 | KEPAVLRN | SEQ ID NO: 5 |
| CVB4 | KEPAVLRN | SEQ ID NO: 5 |
| CVB5 | KEPAVLRN | SEQ ID NO: 5 |
| EVC1 | KEPAVLTK | SEQ ID NO: 6 |
| CVA1 | KEPAVLTK | SEQ ID NO: 6 |
| EVD68 | KEPAVLNS | SEQ ID NO: 7 |
| EVD94 | KEPAVLHS | SEQ ID NO: 8 |
| **3D-2A10-IgG** | | |
| Consensus | $X_1X_2TX_4$VKDELRS$X_{12}X_{13}KX_{15}$ | SEQ ID NO: 2 |
| EV71 BrCr | YSTYVKDELRSLDKI | SEQ ID NO: 9 |

| | | |
|---|---|---|
| EV71 Henan | YSTYVKDELRSLDKI | SEQ ID NO: 9 |
| CA9 | MVTYVKDELRSAEKV | SEQ ID NO: 10 |
| CA16 | YSTYVKDELRSLDKI | SEQ ID NO: 9 |
| CVB1 | MVTYVKDELRSAEKV | SEQ ID NO: 10 |
| CVB3 | MVTYVKDELRSAEKV | SEQ ID NO: 10 |
| CVB4 | MVTYVKDELRSAEKV | SEQ ID NO: 10 |
| CVB5 | MVTYVKDELRSAEKV | SEQ ID NO: 10 |
| EVC1 | LVTYVKDELRSKTKV | SEQ ID NO: 11 |
| CVA1 | LVTYVKDELRSKSKV | SEQ ID NO: 12 |
| EVD68 | FVTFVKDELRSREKV | SEQ ID NO: 13 |
| EVD94 | FVTFVKDELRSREKV | SEQ ID NO: 14 |

**[0043]** Enterovirus is comprised of subtypes A, B, C and D.

**[0044]** Enterovirus A subtype contains 23 serotypes; the exemplary 3D sequences include: (1) human enterovirus 71 (EV71), sub-strain BrCr (GenBank AB204852.1) (SEQ ID NO: 15), where SEQ ID NO: 15 contains the antibody-binding peptides represented by SEQ ID NOS: 3 and 9 respectively; (2) human coxsackievirus A16 strain shzh00-1 (GenBank AY790926.1) (SEQ ID NO: 16), where SEQ ID NO: 16 contains the antibody-binding peptides represented by SEQ ID NOS: 3 and 9 respectively.

**[0045]** Enterovirus B subtype contains 60 serotypes; the exemplary 3D sequences include: (1) human coxsackievirus B3 strain Beijing0811 (GenBank GQ141875.1) (SEQ ID NO: 17), where SEQ ID NO: 17 contains the antibody-binding peptides represented by SEQ ID NOS: 5 and 10 respectively; (2) human coxsackievirus A9 strain Griggs (GenBank D00627.1) (SEQ ID NO: 18), where SEQ ID NO: 18 contains the antibody-binding peptides represented by SEQ ID NOS: 5 and 10 respectively.

**[0046]** Enterovirus C subtype contains 23 serotypes; the exemplary 3D sequences include: (1) human poliovirus 1 isolate CHN-Jiangxi/89-1 (GenBank: AF111984.2) (SEQ ID NO: 19), where SEQ ID NO: 19 contains the antibody-binding peptides represented by SEQ ID NOS: 6 and 11 respectively; (2) human coxsackievirus A1 isolate KS-ZPH01F/XJ/CHN/2011 (GenBank: JX174177.1) (SEQ

ID NO: 20), where SEQ ID NO: 20 contains the antibody-binding peptides represented by SEQ ID NOS: 6 and 12 respectively.

**[0047]** Enterovirus D subtype contains 5 serotypes; the exemplary 3D sequences include: (1) human enterovirus 68 strain Fermon (GenBank: AY426531.1) (SEQ ID NO: 21), where SEQ ID NO: 21 contains the antibody-binding peptides represented by SEQ ID NOS: 7 and 13 respectively; (2) human enterovirus 94 isolate E210 (GenBank: DQ916376.1) (SEQ ID NO: 22), where SEQ ID NO: 22 contains the antibody-binding peptides represented by SEQ ID NOS: 8 and 14 respectively.

**[0048]** Recombinant 3D proteins can be used as immunogens either alone or in combination with other proteins from enteroviruses in immunogenic compositions. As shown in the Examples hereinbelow, 3D protein from EV71 administered together with bacterial flagellin was able to induce antibody responses and partially protect live challenges. Since 3D protein is well conserved in all enteroviruses, it is expected that 3D protein can be used as an immunogen to have broad protections against various subtypes. In addition, certain variants of 3D proteins can also be used in immunogenic compositions, where the identities of these variants in comparison with corresponding wild-type ones are at least 85%, preferably 90%, and more preferably 95%. In addition, the adjuvants may be any known ones such as M59, alum.

**[0049]** Since 3D-2A10-IgG and 3D-3A12-IgG binds to two different epitopes, it would be advantageous to construct a polypeptide that has at least one copy of each of these two epitopes. This 3D protein-derived immunogenic polypeptide can be used in an immunogenic composition for preventing infections of enteroviruses. The cloning and expressing these polypeptides can be performed using well known techniques. In one embodiment, the 3D protein derived immunogenic polypeptide comprises a first peptide represented by SEQ ID NO: 1, a second peptide represented by SEQ ID NO: 2, and an artificial linker, wherein the first and second peptide are covalently coupled by the linker. In certain embodiments, the first and second peptides can have more than one copy, either in tandem or alternate configuration. Any known peptide linker can be used in the present invention, the exemplary linkers

including GGGGS (SEQ ID NO: 23), and TPLGDTTHTSG (Yang JY, Human Vaccines & Immunotherapeutics 2013; 9;1–9) (SEQ ID NO: 24). The linkers in one immunogenic polypeptide can be duplicated or used in tandem.

**[0050]** Since the antibodies against 3D proteins do not show ADE effect, it is advantageous to use an antibody composition to treat patients for enterovirus infection. Antibody compositions for therapeutic uses are well known such as antibodies for cancer treatment. The antibody compositions usually comprise pharmaceutically acceptable ingredients such as sodium chloride dissolved in a pharmaceutically acceptable solution.

**[0051]** The following examples are provided for the sole purpose of illustrating the principles or implementation of the present invention; they are by no means intended to limit or narrow the scope of the present invention.

**[0052]** Example 1

**[0053]** EV71 3D-specific IgG monoclonal antibodies

**[0054]** 1.1. Antigen preparation

**[0055]** Complete *3D* gene (encoded protein as shown in SEQ ID NO: 15) of EV71 BrCr strain was cloned into vector pET28a with NcoI and XhoI. Briefly, the transformed bacteria *E. coli* BL21(DE3) containing recombinant 3D expression constructs were grown and induced. These recombinant proteins were prepared and purified by affinity chromatography on a Ni-NTA column (Qiagen).

**[0056]** 1.2. Immunization and production of EV71 3D-specific IgG monoclonal antibodies

**[0057]** The method for generating EV71 3D-specific mAbs was as previously described (Li YM, Liu F, Han C and Yan HM. Monoclonal antibody that blocks the Toll-like receptor 5 binding region of flagellin. Hybridoma (Larchmt). 2012 Feb;31(1):60-62). Briefly, 5-week-old female SPF BALB/c mice were immunized subcutaneously with 100μg 3D at 2-week interval. Four weeks after the last booster and 3 days before cell fusion, the mice were boosted with 200μg of 3D i.p. Three days later, murine splenocytes were harvested and fused with SP2/0 using 50% polyethyleneglycol (Sigma-Aldrich, MO). Hybridoma culture supernatants were

screened using ELISA. The positive hybridoma cells were cloned by a limiting dilution and the stable hybridoma clones were injected into liquid paraffin-pretreated abdominal cavities of BALB/c mice. Subsequently, the MAbs were harvested and purified from the seroperitoneum with an antibody purification kit according to the manufacturer's specifications (NAb™ Protein A/G Spin Kit, Thermo Scientific, USA).

**[0058]** 1.3. Verification of the binding specificities of 3D-specific IgG mAbs

**[0059]** Vero-1008 cells in 24-well plate were infected with EV71 (MOI = 0.1). At 24 hours post-infection, cells were fixed with absolute methanol and processed for indirect immunofluorescence assay (IFA) using indicated 3D-specific MAbs (3A12, 2A10, 7A6G1, and 11F1), followed by fluorescein isocyanate-conjugated goat anti-mouse IgG antibodies; a flagellin-specific Mab (5G10) was included as a negative control, and 3D-immunized mice serum as positive control. As shown in FIG 1(A), 3A12, 2A10, 7A6G1 and 11F1 showed evident staining to a level comparable to the positive control, but negative control MAb 5G10 showed no staining.

**[0060]** Vero-1008 cells were cultured and infected as above described. Cell lysates were separated by SDS-PAGE, and transferred to PVDF membrane that was blotted by indicated antibodies following conventional western blot protocol. As shown in FIG 1(B), lane 1, 3A12; lane 2, 2A10; lane 3, 7A6G1; lane 4,11F1; lane 5, 5G10 as negative control; and lane 6, positive control. The result showed that 3D protein-specific IgG mAbs (3A12, 2A10, 7A6G1 and 11F1) revealed a specific band corresponding to 3D protein, but negative control (5G10) showed no binding to 3D.

**[0061]** 1.4. Intracellular inhibition of EV71 replication by 3D-specific IgG mAbs

**[0062]** Vero-1008 cells were seeded in 24-well plate at $1 \times 10^5$ cells per well and cultured for 24 hours, and then infected with EV71 at MOI = 0.1. At 1 hour post-infection, cell supernatants were removed and the cells were washed 3 times. 5μg IgG antibodies diluted in OPTI-MEM was added to 100 μl OPTI-MEM with or

without 5 μl Lipofectamine 2000, and the mixture was added to virus-infected cells. Then, at 3 hours post-transfection, cell supernatants were replaced with 0.5 ml DMEM with 3% FBS. Eight hours later, EV71-infected cells were obtained and undergone one freeze-thaw cycle, and the virus titers (represented by PFU/well) in these cell samples were titrated by plaque assay. As show in FIG 2(A), the transfected 3A12 and 2A10 significantly reduced the virus titers; the transfected 7A6G1 reduced the virus titer, but less significant than 3A12 and 2A10; but the transfected 11F1 failed to reduce the virus titer. As for EV-5, it was a VP2-specific IgG mAb; the transfected EV-5 failed to reduce the virus titer; it was noteworthy that EV-5, when directly added into cell culture, showed increased virus titer, suggesting an effect of antibody-dependent enhancement (ADE). The data of the virus titers demonstrated that the epitopes recognized by different 3D-specific IgG mAbs were important for their capability of inhibiting EV71 replication.

**[0063]** Vero-1008 cells were cultured and infected and treated with antibodies as above described except for the doses of antibodies. As shown in FIG 2(B), the transfected 2A10 exhibited a dose-dependent inhibitory effect on the reduction of virus titers within the tested doses (0, 0.2, 1 or 5 μg/well), but the transfected EV-5 showed no inhibition at all tested doses. Three independent experiments were performed in duplicate, and the representative data were presented.

**[0064]** 1.5. 3D-specific IgG mAbs inhibited *in vitro* 3D polymerase activities

**[0065]** As shown in FIG 3(A), there is provided a schematic diagram of 3D (RdRp)-mediated RNA elongation.

**[0066]** In 10 μl reaction system (50 mM HEPES, 75 mM KCl, 5 mM $MgCl_2$, 4 mM TCEP, 300 μM NTP, and 4 μM RNA Complex), the addition of 1 μg functional 3D triggered initiation and elongation of the RNA. RNA species were resolved by 15% polyacrylamide gel electrophoresis including 7 M Urea and visualized by Stains-All staining. For inhibition assays, 2μg IgG mAbs were added in each reaction. RNA elongation activity of 3D could be determined based on the appearance of elongated

RNA band. As shown in FIG 3(B), lane 1, negative control without polymerase; lane 2, positive control (no interventional factor); lane 3, 3A12; lane 4, 2A10; lane 5, 7A6G1; lane 6,11F1; and lane 7, EV-5. 3A12, 2A10, and 7A6G significantly suppressed the appearance of the elongated RNA band, but 11F1 and EV-5 failed to suppress the appearance of the elongated RNA band. Three independent experiments were performed, and the representative data were presented.

**[0067]** 1.6. Antiviral efficacy of 3D-specific MAbs in an *in vivo* murine model

**[0068]** Antiviral efficacy of EV71 3D-specific IgG was investigated in an in vivo murine model. 30 one-day old neonatal mice were randomly divided into 6 groups (5 mice per group). Each group received, ip, 100 μg/50 μl 3A12, 2A10, 7A6G1, 11F1 or EV-5 IgG antibodies respectively, and PBS group was include as negative control; then each group received $10^3$ $TCID_{50}$ EV71 challenge i.p. And IgG was injected with 24-hours interval for 4 times. The mice survival data were collected each day for 2 weeks. As shown in FIG 4, 2A10-IgG and 3A12-IgG conferred 20% or 40% protection respectively.

**[0069]** 1.7. *In vitro* virus replication with the presence of 2A10-IgG or EV-5-IgG

**[0070]** $1 \times 10^4$ PFU EV71 were added to the consecutively diluted 200 μl mAbs (EV-5 IgG for EV71 VP2, or 2A10-IgG for EV71 3D). The mixture infected the Caco-2 cell after 1 hour incubation. The EV71-infected Caco-2 cells were harvested, and the virus titers in these cell samples were titrated by plaque assay. As shown in FIG 5, EV-5 significantly enhanced the viral infection under the concentrations between 0.25-16μg/ml compared with the baseline ($P < 0.01$), but 2A10 did not enhance the viral infection at all tested doses.

**[0071]** 1.8. Mapping the epitopes of EV71 3D-specific 3A12 and 2A10 and its space situation in three dimension model of EV71 3D (1RA6)

**[0072]** *3D* gene (encoded protein represented by SEQ ID NO: 15) and its truncated mutants were cloned into the pET28a expression vector, and the binding

activity of IgAs to 3D protein and mutants was determined by Western blot. Furthermore, synthesized distinct lengths of peptides were used to identify the exact domain recognized by 3A12-IgG and 2A10-IgG. As shown in Table 1, 3A12 and 2A10 recognized the polypeptides: KEPAVLTS (SEQ ID NO: 3) and YSTYVKDELRSLDKI (SEQ ID NO: 9), respectively. An alignment of various enterovirus strains from all A, B, C and D subtypes revealed one consensus sequence $KEPAVLX_7X_8$ (SEQ ID NO: 1) recognized by 3A12, and another consensus sequence $X_1X_2TX_4VKDELRSX_{12}X_{13}KX_{15}$ (SEQ ID NO: 2) recognized by 2A10. As shown in FIG 6, EV71 3D (1RA6) was used to indicate position of the two identified epitopes of 3A12 (orange) and 2A10 (light teal).

**[0073]** 1.9. Intracellular inhibition of the replication of EV71 and CV strains by EV71 3D-specific IgAs

**[0074]** Since IgA monoclonal antibodies were convenient for assaying intracellular inhibition of different strains, 3A12-IgG and 2A10-IgG have been subclass-switched into corresponding IgA mAbs, 3A12-IgA and 2A10-IgA. The details are included in the co-pending application entitled "**3D PROTEIN-SPECIFIC MONOCLONAL IMMUNOGLOBULIN A ANTIBODIES**", which is incorporated herein in its entirety.

**[0075]** The results of intracellular inhibition of the replication of EV71 and CV strains were summarized hereinbelow in Table 2.

**[0076]** Table 2. Intracellular inhibition of the replication of EV71 and CV strains

| | MAb | | | |
|---|---|---|---|---|
| | 3A12-IgA | 2A10-IgA | 16CF7-IgA | Media alone |
| Virus clades | Virus titer (PFU/well) /(%Reduction of virus[a]) | Virus titer (PFU/well) /(%Reduction of virus[a]) | Virus titer (PFU/well) /(%Reduction of virus[a]) | Virus titer (PFU/well) /(%Reduction of virus[a]) |
| EV71 | 11400 /(81.3) | 23600 /(61.5) | 57300 /(6.4) | 61200 /(0) |
| CVB1 | 31500 /(46.4) | 34600 /(41.1) | 59400 /(-1.2) | 58700 /(0) |
| CVB3 | 31200 | 40500 | 71300 | 73400 |

| | | | | |
|---|---|---|---|---|
| | /(57.5) | /(44.8) | /(2.9) | (0) |
| CVB4 | 21000 /(58.8) | 25700 /(49.5) | 47700 /(6.3) | 50900 /(0) |
| CVB5 | 18500 /(67.8) | 18400 /(67.9) | 54800 /(4.4) | 57300 /(0) |
| CVA9 | 21300 /(58.5) | 27100 /(47.2) | 49300 /(3.9) | 51300 /(0) |

**[0077]** where “a” denotes that “%Reducton of virus” was calculated after the percentages of virus survivals in the presence of different antibodies were calculated using the virus titers in the media alone groups as 100%.

**[0078]** Example 2

**[0079]** 3D as an antigen

**[0080]** 2.1. Identification of 3D in recombinant Vaccinia virus Tiantan strain expressing 3D (VTT-3D)

**[0081]** EV71 3D was detected in recombinant Vaccinia Virus-infected Vero cells. As shown in FIG 7, Lane 1, protein molecular weight marker, lane 2, mock infected Vero control, lane 3, VTTenv infected Vero, lane 4-7, four purified recombinant Vaccinia Virus clone-infected Vero. The infected or non-infected Vero were separated by SDS-PAGE, and transferred to PVDF membrane. 3D-specific IgG mAb 2A10-IgG was used as binding antibody. The result show that 3D gene inserted into Vaccinia Virus genome and expressed correctly in Vero cell.

**[0082]** 2.2. Immunization program

**[0083]** Table 3. Immunization program

| Groups | Immunogen | Route of immunization | No. of mice |
|---|---|---|---|
| 1 | 3D, 3D, 3D 30μg | 2S.C.+I.N. | 6 |
| 2 | VTT-3D, $10^7$pfu 3D,VTT-3D | 2S.C.+I.N. | 6 |

**[0084]** As shown in FIG 8, there is provided a schematic diagram showing the immunization protocol.

**[0085]** Antibody responses were assayed after each immunization: prime (FIG 9); first boost (FIG 10); second boost (FIG 11). Each mouse in VTT-3D group

received immunization of $10^7$ PFU of virus in 100 μl, whereas each mouse in 3D group was subject to be immunized with 30 μg 3D protein in 100 μl. Serum and mucosal samples from vagina and saliva were collected and subject to determine the titer of IgA against 3D protein by ELISA.

**[0086]** Antibody responses were assayed in neonatal mice (FIG 12).

**[0087]** 2.3. Protection against EV71 challenge

**[0088]** After three times immunization, each neonatal mouse in VTT-3D group, 3D group, PBS groups as well as inactivated EV71 group was challenged with $10^3$ TCID50 of virus. Neonatal mouse was observed each day. Inactivated EV71 immunization conferred complete protection of mice. All the mice in PBS negative control died in 3-5 days after challenge. As shown in FIG 13, 3D and VTT-3D conferred mice 10%-30% protection from challenge.

**[0089]** Example 3

**[0090]** 3.1. Immunization with purified 3D proteins

**[0091]** The expression and purification of 3D protein were described in Example 1.1. Purified 3D protein was adjuvanted with flagellin from *E coli.* or CTB, and then immunized mice subcutaneously (SC), intranasally (IN) or intraperitoneally (IP). The immunization protocol was summarized in Table 4. The intervals between two immunizations were 2 weeks; the volumes for SC and IP were 100μl, while the volumes for IN were 20μl.

**[0092]** Table 4. Immunization protocol with purified 3D proteins

| Groups | Immune compositions | Immunization methods | Number of mice per group |
|---|---|---|---|
| 1 | 30μg3D+5μgKF | 2xSC+2xIN | 6 |
| 2 | 30μg3D+5μgKF | 2xIP+2xIN | 6 |
| 3 | 30μg3D+2μgCTB | 2xSC+2xIN | 6 |
| 4 | 30μg3D+2μgCTB | 2xIP+2xIN | 6 |
| 5 | PBS | 2xSC+2xIN | 6 |

**[0093]** 3.2. Antibody responses

**[0094]** 2-weeks after the final immunization, sera, intestine and lung samples were collected; the intestine and lung samples were homogenized and supernatants

were collected for testing. 3D-specific IgA or IgG antibody responses in these samples were titrated by ELISA. FIG 14 shows the titers of 3D-specific IgG antibodies in serum (a), lung (b) and intestine (c), and 3D-specific IgA antibodies in serum (d), lung (e) and intestine (f).

**[0095]** While the present invention has been described with reference to particular embodiments, it will be understood that the embodiments are illustrative and that the invention scope is not so limited. Alternative embodiments of the present invention will become apparent to those having ordinary skill in the art to which the present invention pertains. Such alternate embodiments are considered to be encompassed within the scope of the present invention. Accordingly, the scope of the present invention is defined by the appended claims and is supported by the foregoing description.

Print Out (Original in Electronic Form)

| | | |
|---|---|---|
| **0-1** | **Form PCT/RO/134 (SAFE) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)** | |
| 0-1-1 | Prepared Using | **CEPCT Version 1.01.00 MT/FOP 20140331/0.20.5.21** |
| **0-2** | **International Application No.** | PCT/CN2015/071022 |
| **0-3** | **Applicant's or agent's file reference** | **00101** |

| | | |
|---|---|---|
| **1** | **The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:** | |
| **1-1** | **page** | **0045** |
| **1-2** | **line** | |
| **1-3** | **Identification of deposit** | |
| 1-3-1 | Name of depositary institution | **China Center for Type Culture Collection** |
| 1-3-2 | Address of depositary institution | **Wuhan University,Hubei Privince,China** |
| 1-3-3 | Date of deposit | **22 July 2014 (22.07.2014)** |
| 1-3-4 | Accession Number | **CCTCC C2014141** |
| **1-4** | **Additional Indications** | |
| **1-5** | **Designated States for Which Indications are Made** | **All designations** |
| **1-6** | **Separate Furnishing of Indications** These indications will be submitted to the International Bureau later | |
| **2** | **The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:** | |
| **2-1** | **page** | **0045** |
| **2-2** | **line** | |
| **2-3** | **Identification of deposit** | |
| 2-3-1 | Name of depositary institution | **China Center for Type Culture Collection** |
| 2-3-2 | Address of depositary institution | **Wuhan University,Hubei Privince,China** |
| 2-3-3 | Date of deposit | **22 July 2014 (22.07.2014)** |
| 2-3-4 | Accession Number | **CCTCC C2014143** |
| **2-4** | **Additional Indications** | |
| **2-5** | **Designated States for Which Indications are Made** | **All designations** |
| **2-6** | **Separate Furnishing of Indications** These indications will be submitted to the International Bureau later | |

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| **0-4** | **This form was received with the international application:** (yes or no) | |
| 0-4-1 | Authorized officer | |

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|---|---|---|
| **0-5** | **This form was received by the international Bureau on:** | |
| 0-5-1 | Authorized officer | |

# CLAIMS

What is claimed is:

1. A monoclonal immunoglobulin G (IgG) antibody specifically binding to a peptide, wherein the peptide is represented by a consensus sequence selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO: 2.

2. The monoclonal IgG antibody of claim 1, wherein the monoclonal IgG antibody binding to the peptide represented by SEQ ID NO: 1 is 3D-2A10-IgG (CCTCC NO: C2014143), and the monoclonal IgG antibody binding to the peptide represented by SEQ ID NO: 2 is 3D-3A12-IgG (CCTCC NO: C2014141).

3. The monoclonal IgG antibody of claim 1, wherein the monoclonal IgG antibody is selected from the group consisting of an antibody comprising an Fc domain, a single-chain antibody, and a Fab fragment.

4. The monoclonal IgG antibody of claim 1, wherein the peptide represented by SEQ ID NO: 1 is selected from the group consisting of the peptides represented by SEQ ID NOS: 3-8, and the peptide represented by SEQ ID NO: 2 is selected from the group consisting of the peptides represented by SEQ ID NOS: 10-14.

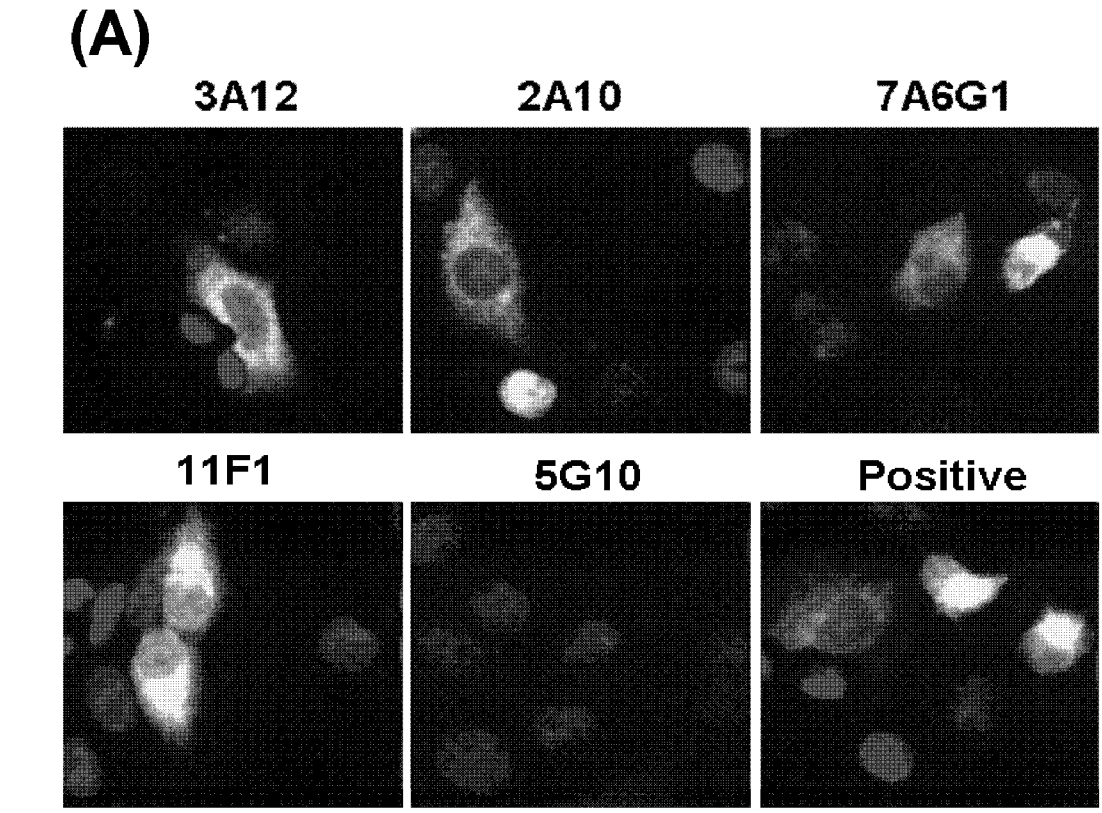
5. An immunogenic composition, comprising a recombinant 3D protein or a 3D protein derived immunogenic polypeptide, and a pharmaceutically acceptable adjuvant.

6. The immunogenic composition of claim 5, wherein the recombinant 3D protein is selected from the group consisting of the 3D proteins represented by SEQ ID NOS: 15-22, and variants with at least 85% identity to corresponding wild-type 3D proteins.

7. The immunogenic composition of claim 5, wherein the 3D protein derived immunogenic polypeptide comprises a first peptide represented by SEQ ID NO: 1, a second peptide represented by SEQ ID NO: 2, and an artificial linker, wherein the first and second peptide are covalently coupled by the linker.

8. The immunogenic composition of claim 7, wherein the first peptide is selected from the group consisting of the peptides represented by SEQ ID NOS: 3-8, and the second peptide is selected from the group consisting of the peptides represented by SEQ ID NOS: 10-14.

9. The immunogenic composition of claim 7, wherein the artificial linker is selected from the group consisting of the linkers represented by SEQ ID NOS: 23 and 24.



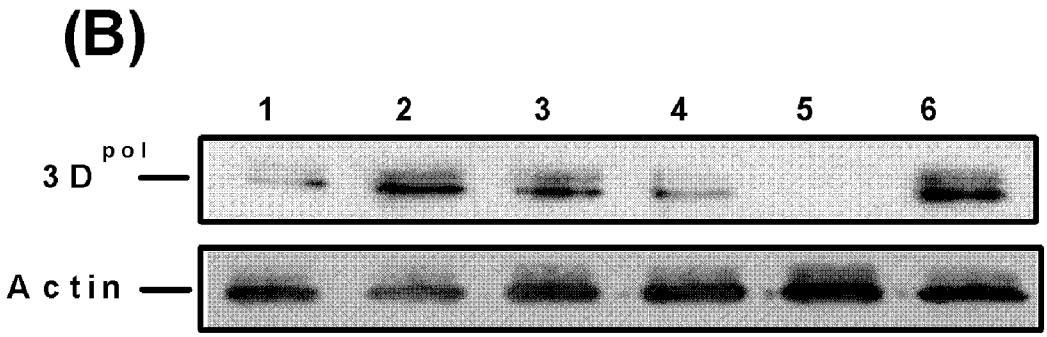




FIG 1

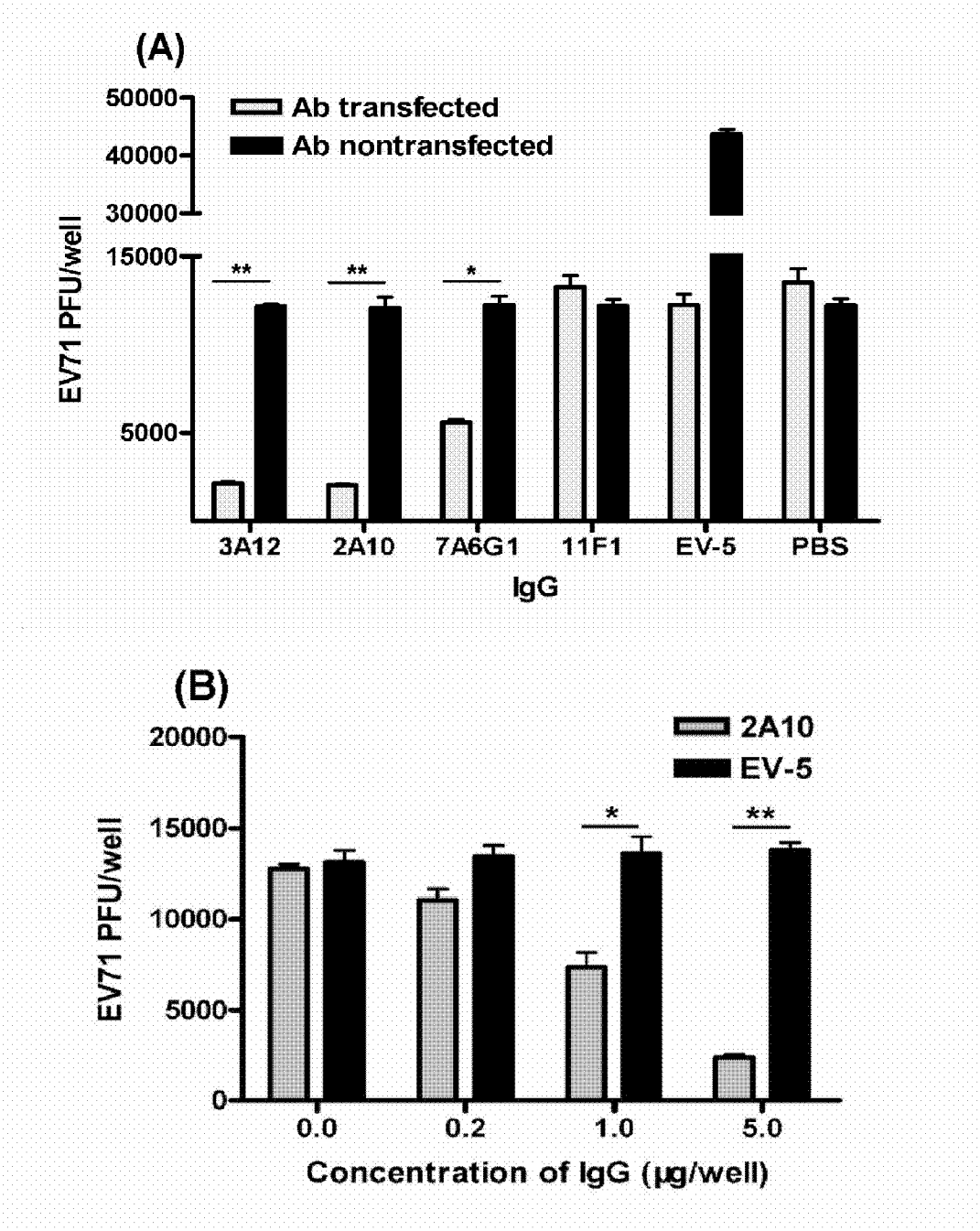

(A)
Ab transfected
Ab nontransfected
EV71 PFU/well
50000
40000
30000
15000
5000
**
**
*
3A12
2A10
7A6G1
11F1
EV-5
PBS
IgG
(B)
2A10
EV-5
EV71 PFU/well
20000
15000
10000
5000
0
*
**
0.0
0.2
1.0
5.0
Concentration of IgG (μg/well)

FIG 2

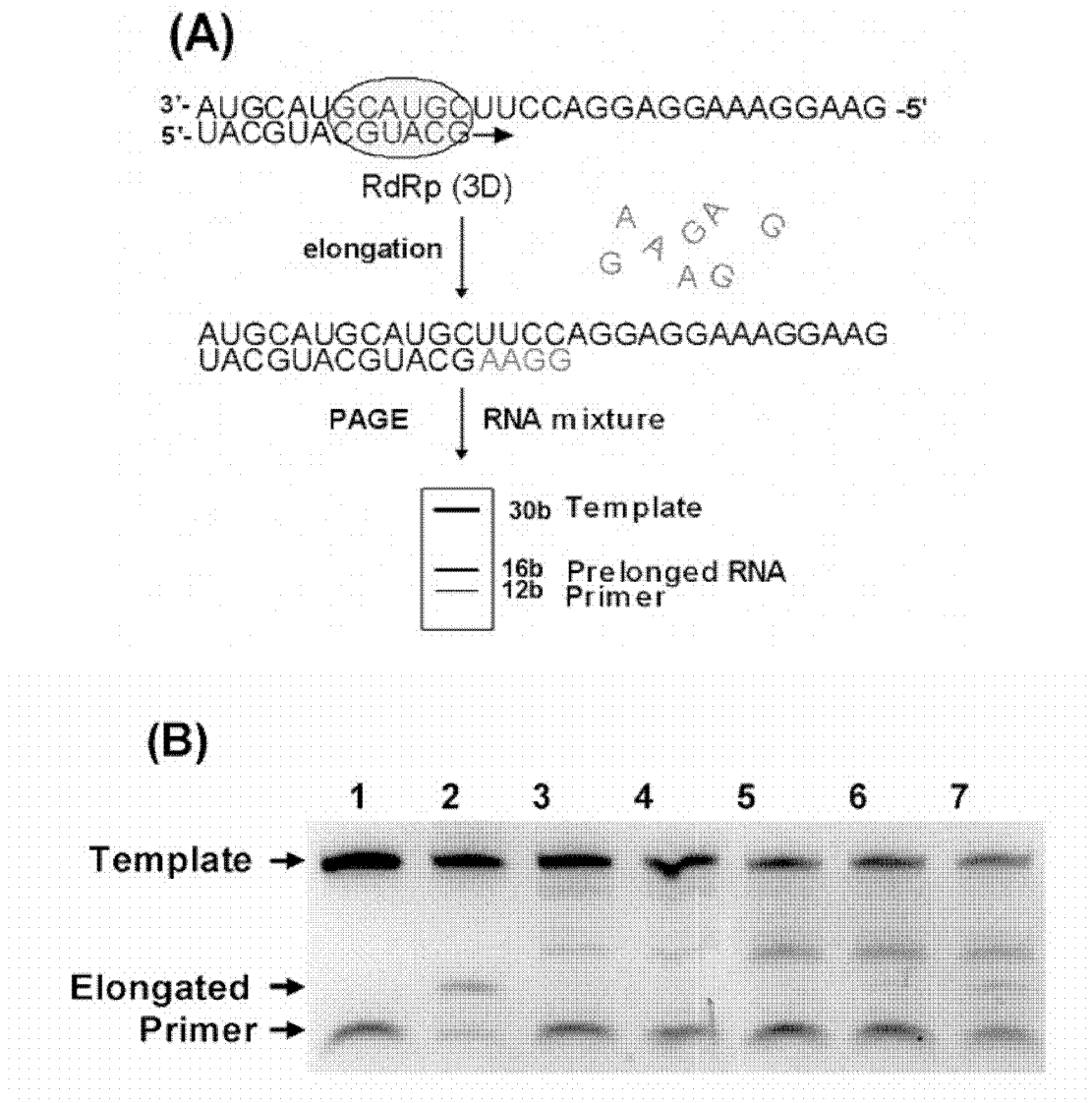

(A)
3'- AUGCAUGCAUGCUUCCAGGAGGAAAGGAAG -5'
5'- UACGUACGUACG
RdRp (3D)
elongation
AUGCAUGCAUGCUUCCAGGAGGAAAGGAAG
UACGUACGUACGAAGG
PAGE
RNA mixture
30b Template
16b Prelonged RNA
12b Primer
(B)
1 2 3 4 5 6 7
Template
Elongated
Primer

FIG 3

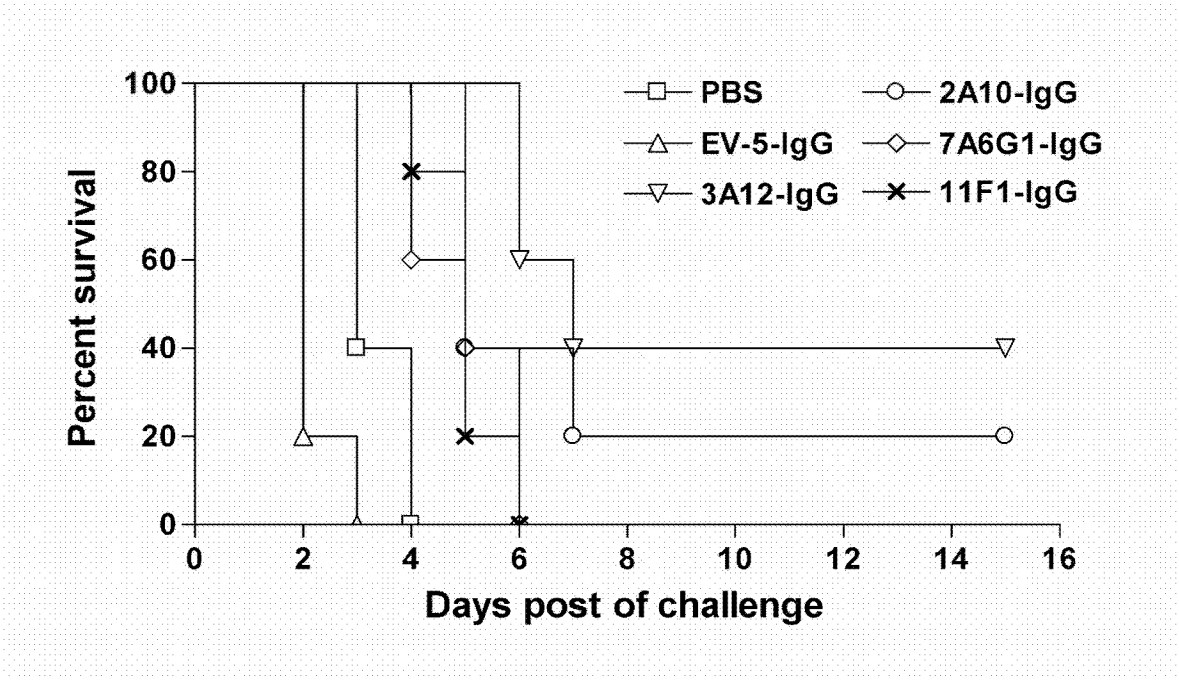

Percent survival
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
Days post of challenge
PBS
2A10-IgG
EV-5-IgG
7A6G1-IgG
3A12-IgG
11F1-IgG

FIG 4

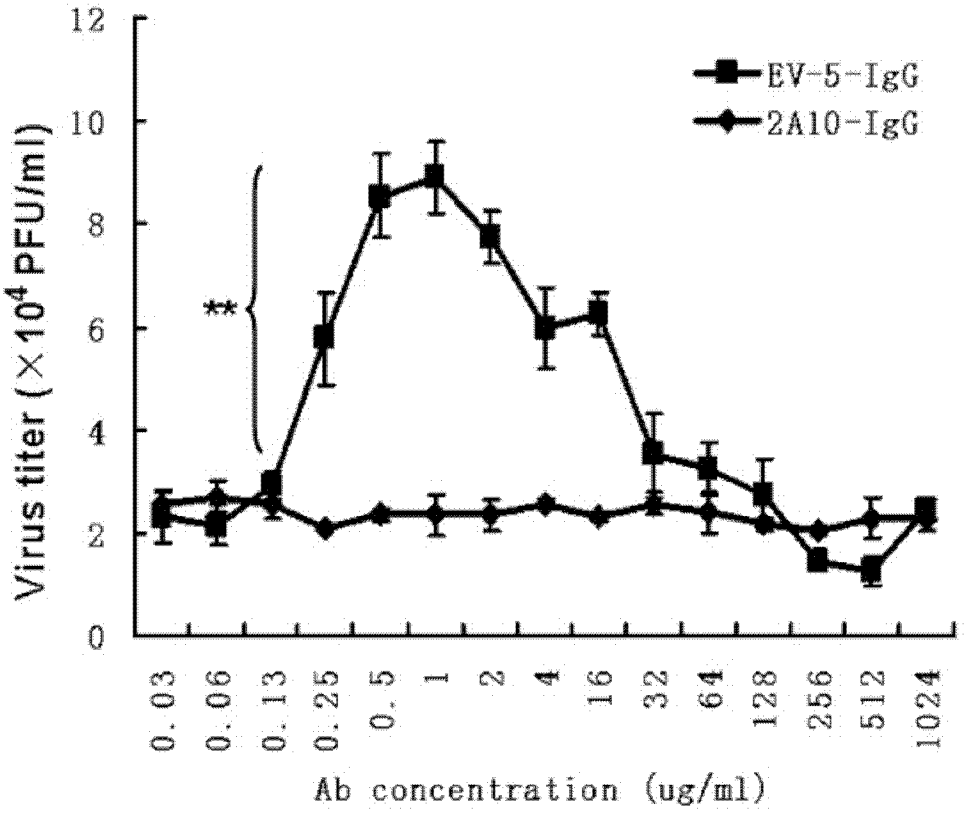

12
10
8
6
4
2
0
Virus titer (×10⁴ PFU/ml)
EV-5-IgG
2A10-IgG
**
0.03
0.06
0.13
0.25
0.5
1
2
4
16
32
64
128
256
512
1024
Ab concentration (ug/ml)

FIG 5

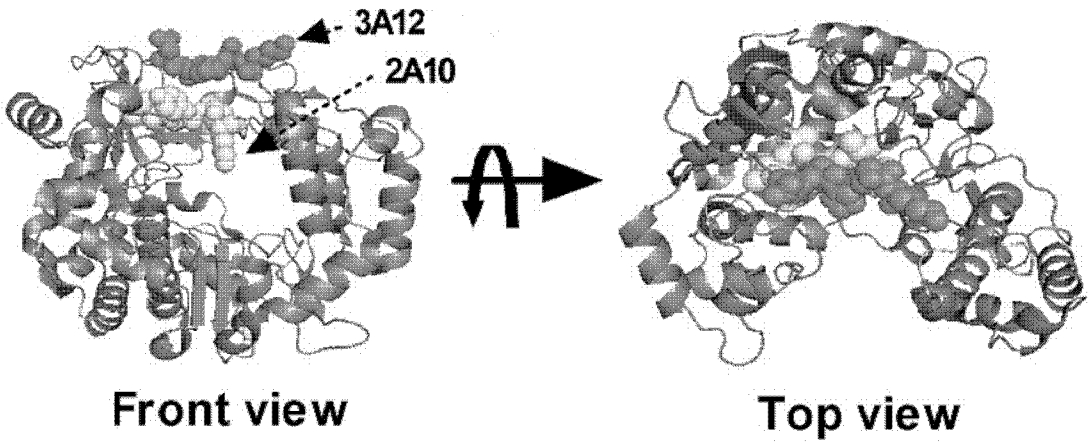



FIG 6

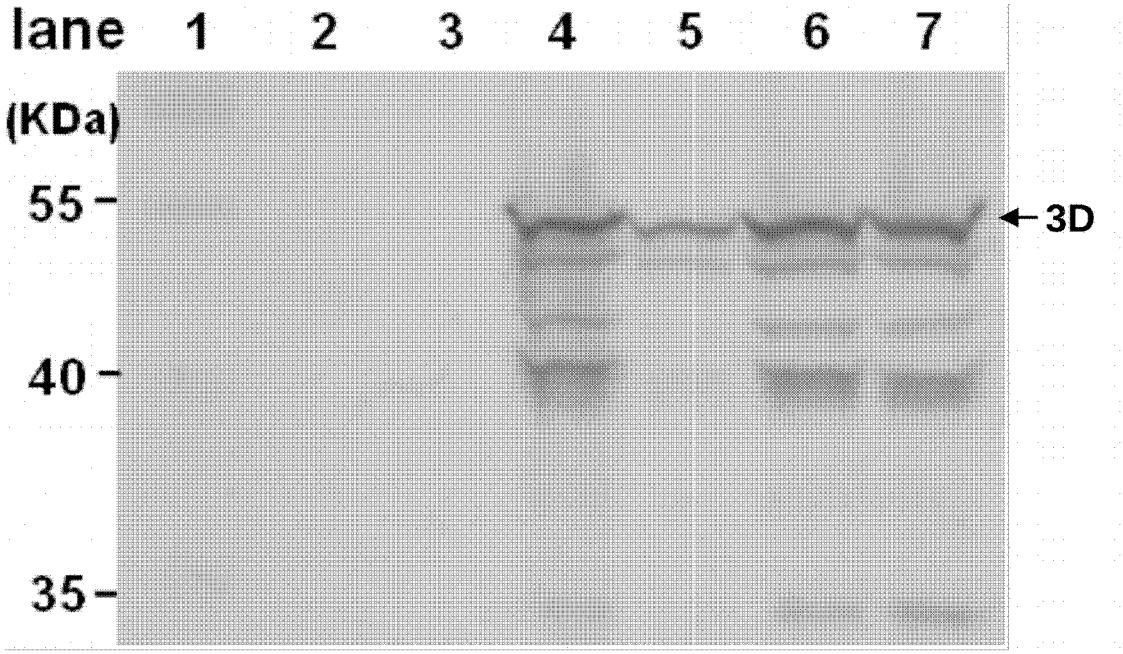



FIG 7

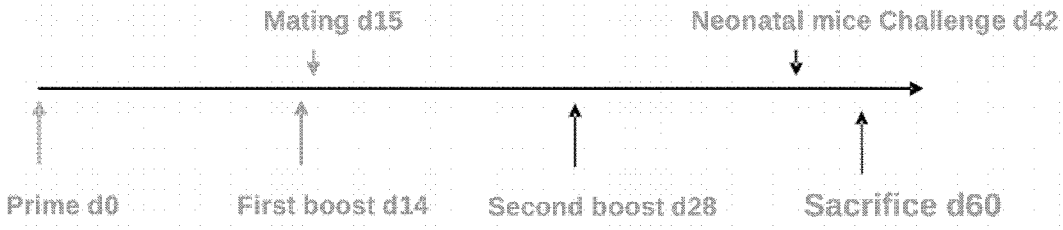
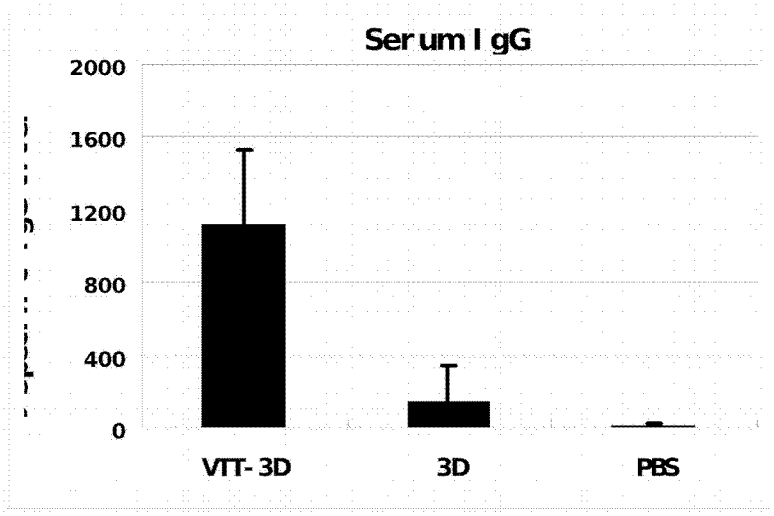
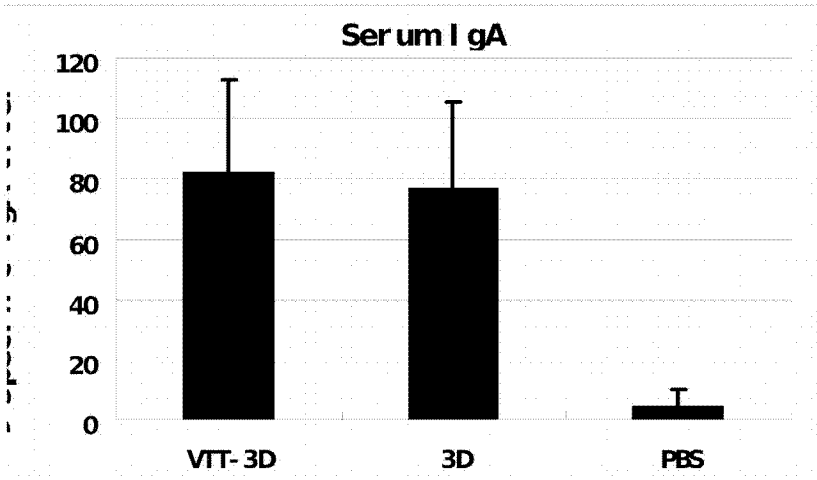

Mating d15
Neonatal mice Challenge d42
Prime d0
First boost d14
Second boost d28
Sacrifice d60

FIG 8

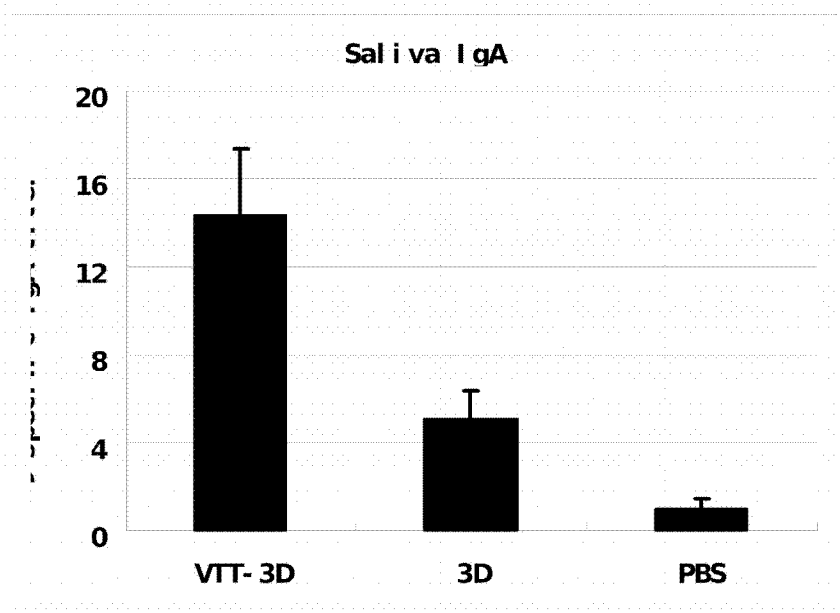

Serum IgG
2000
1600
1200
800
400
0
VTT-3D
3D
PBS

(a)

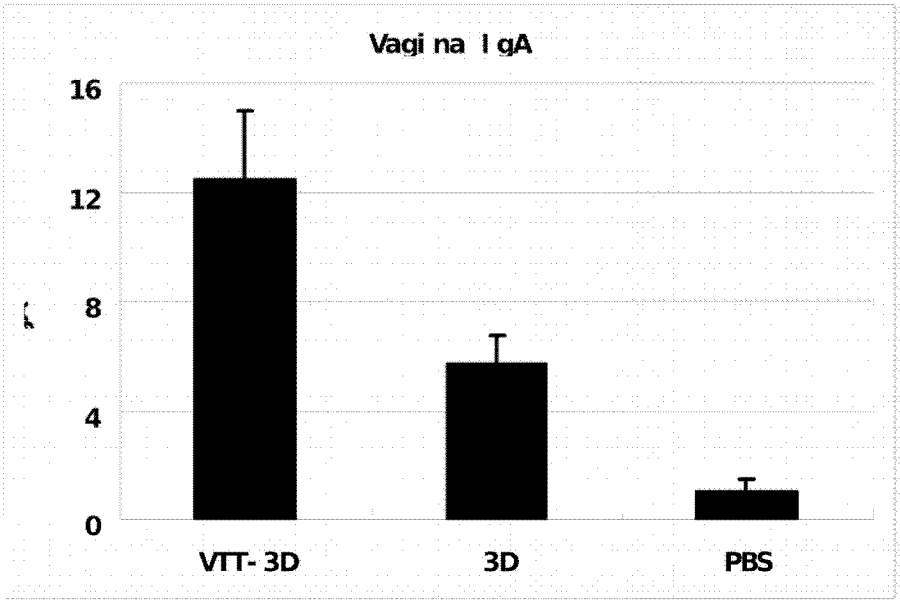

Serum IgA
120
100
80
60
40
20
0
VTT-3D
3D
PBS

(b)

FIG 9

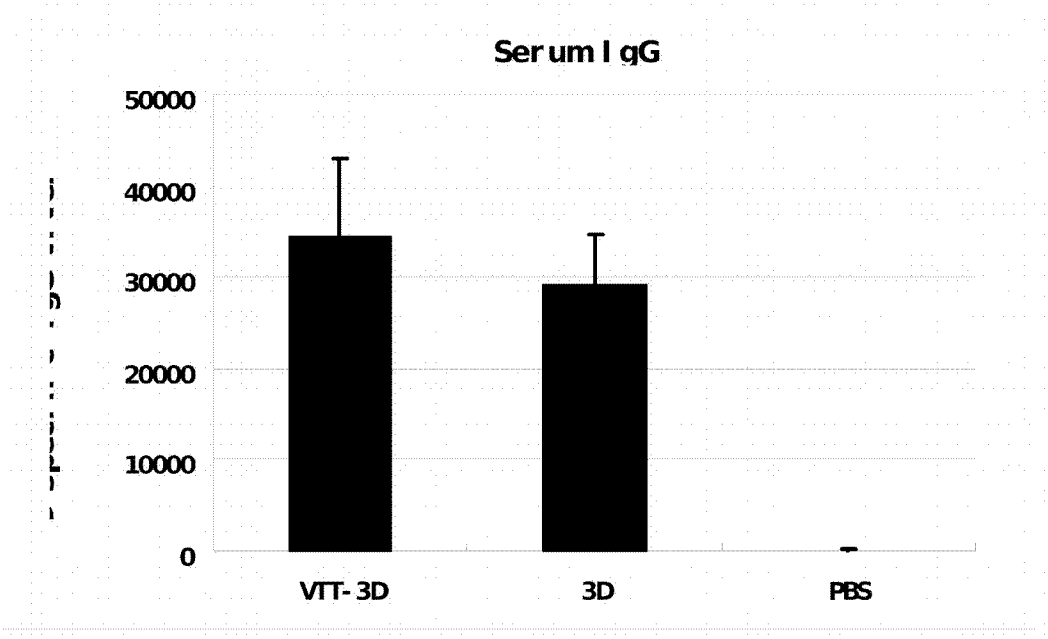



(c)

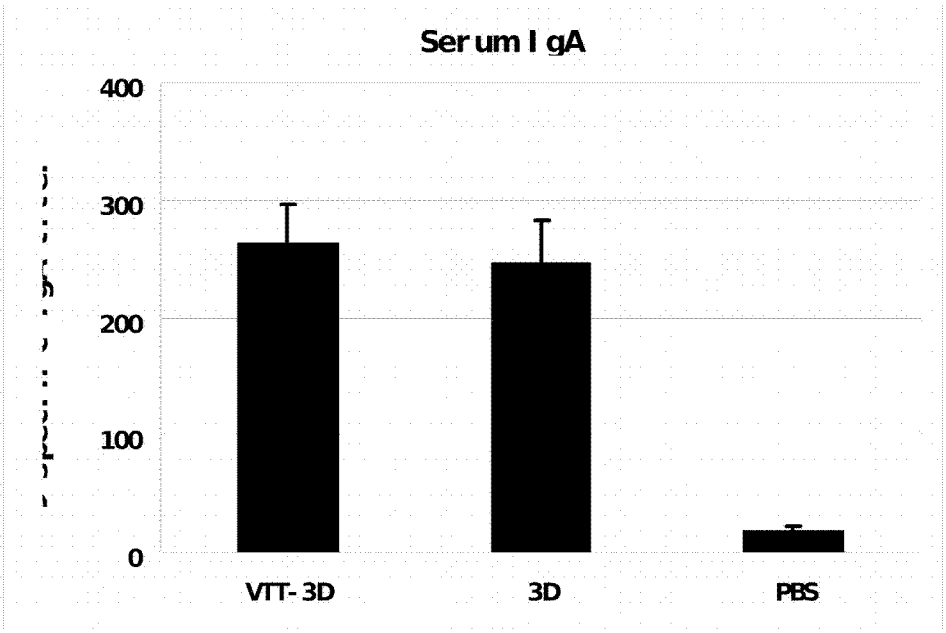



(d)

**FIG 9 (cont'd)**

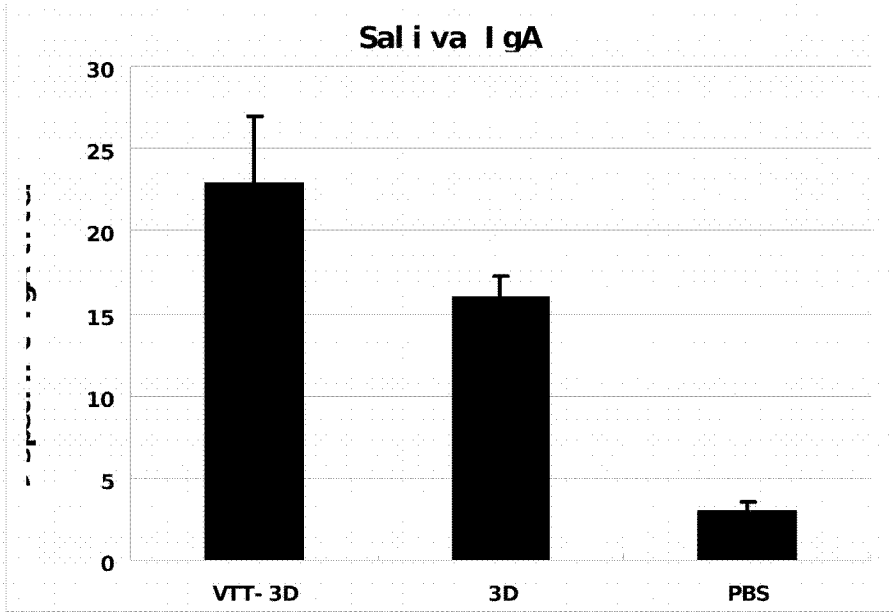

Serum IgG
50000
40000
30000
20000
10000
0
VTT-3D
3D
PBS

(a)

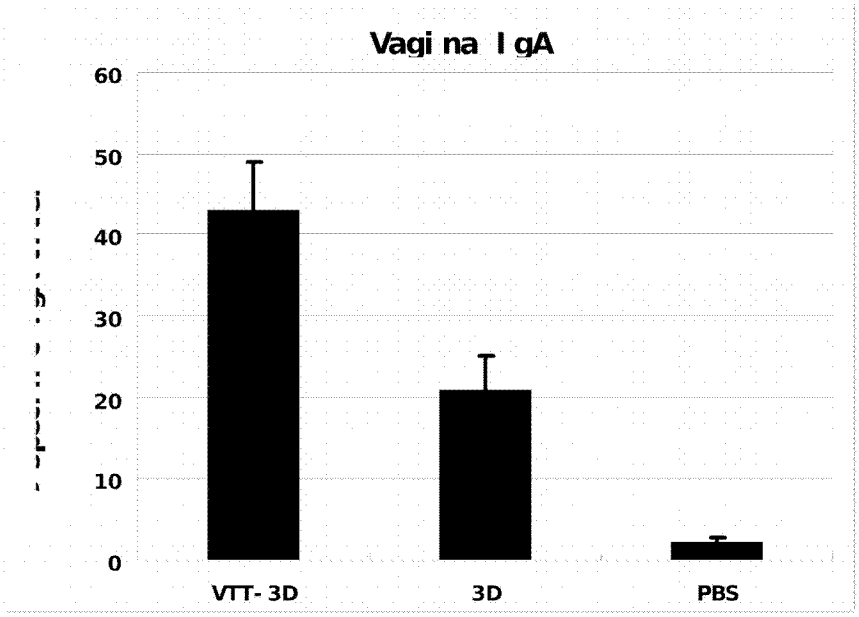

Serum IgA
400
300
200
100
0
VTT-3D
3D
PBS

(b)

FIG 10

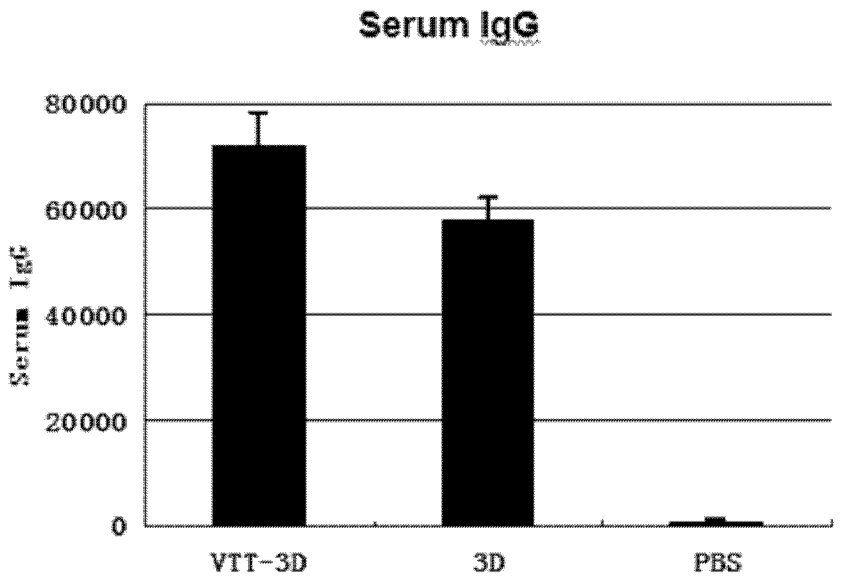



(c)

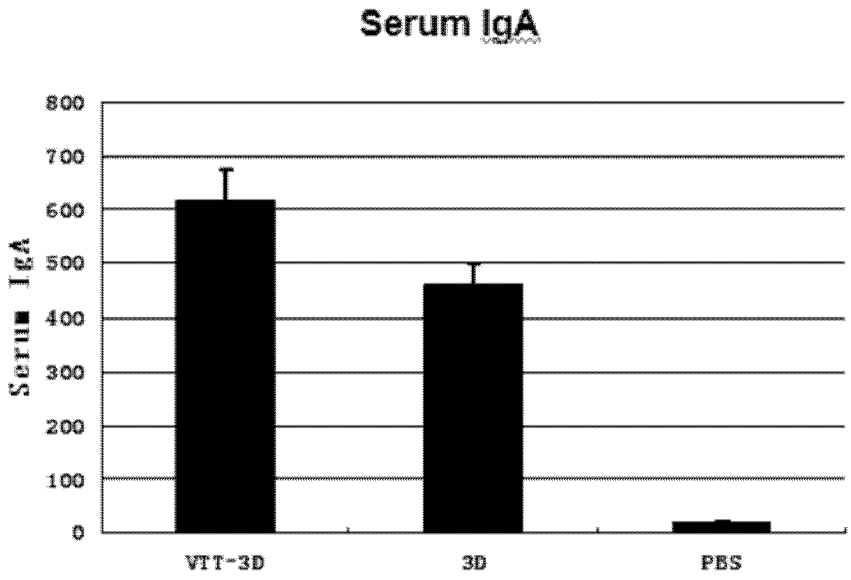



(d)

FIG 10 (cont'd)

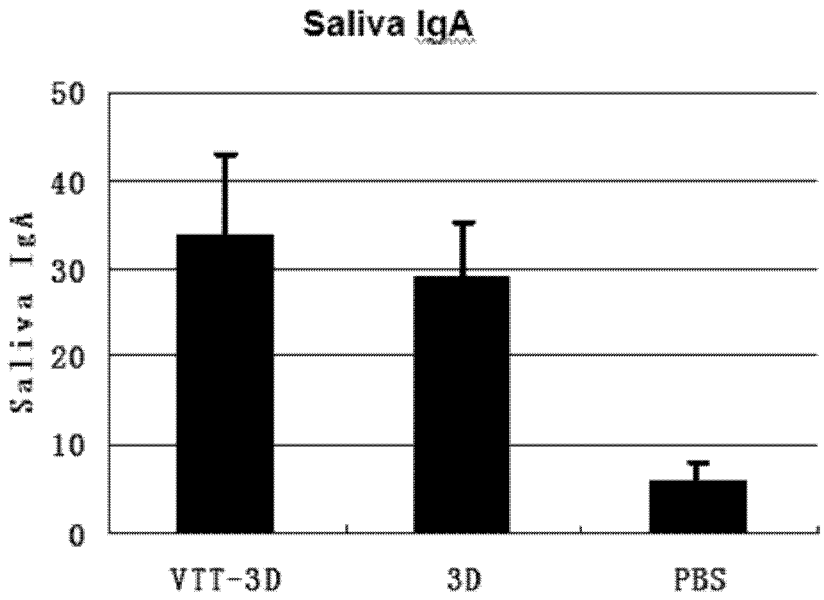

Serum IgG
80000
60000
40000
20000
0
Serum IgG
VTT-3D
3D
PBS

(a)

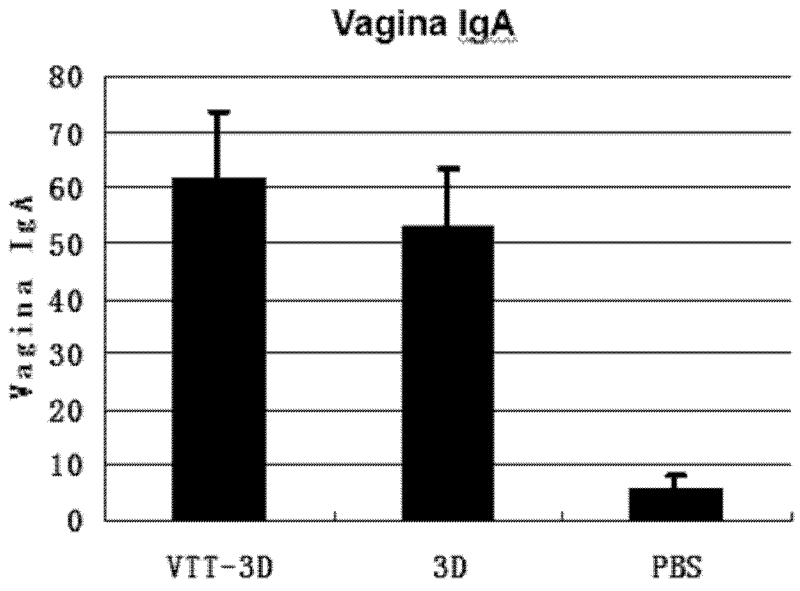

Serum IgA
800
700
600
500
400
300
200
100
0
Serum IgA
VTT-3D
3D
PBS

(b)

FIG 11




(c)




(d)

**FIG 11 (cont'd)**

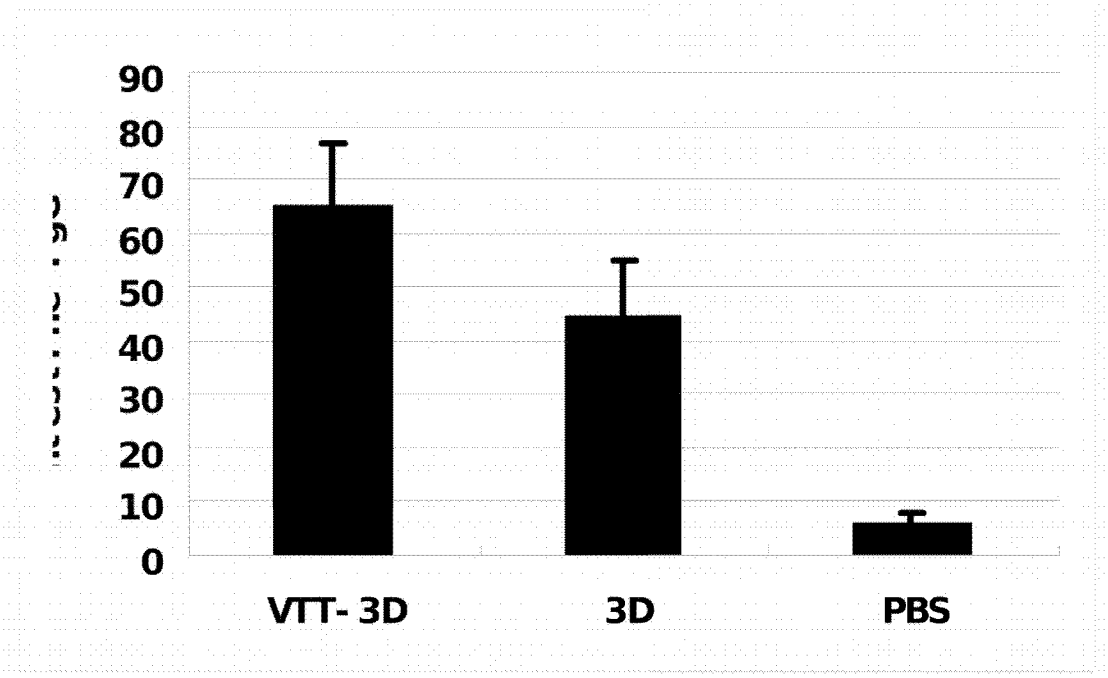



FIG 12

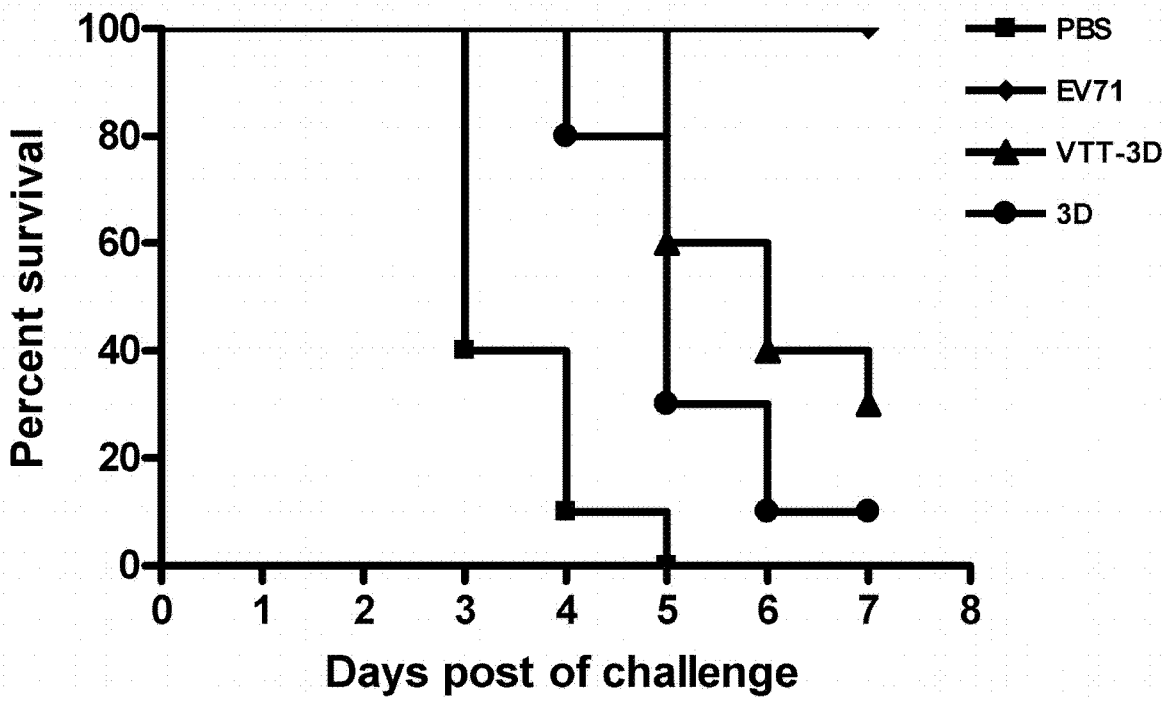
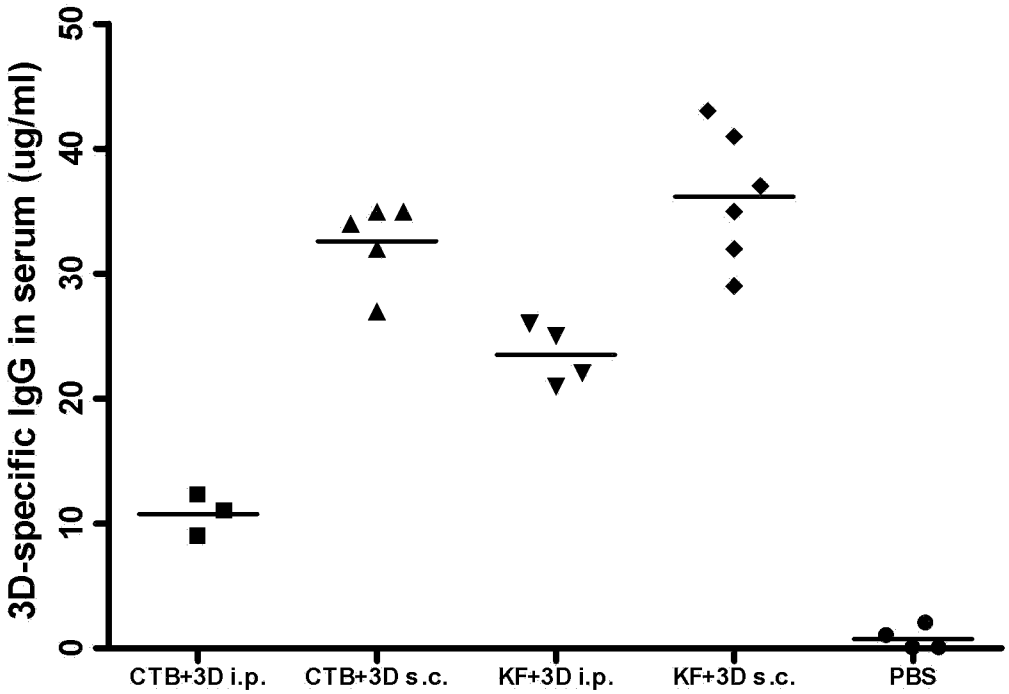
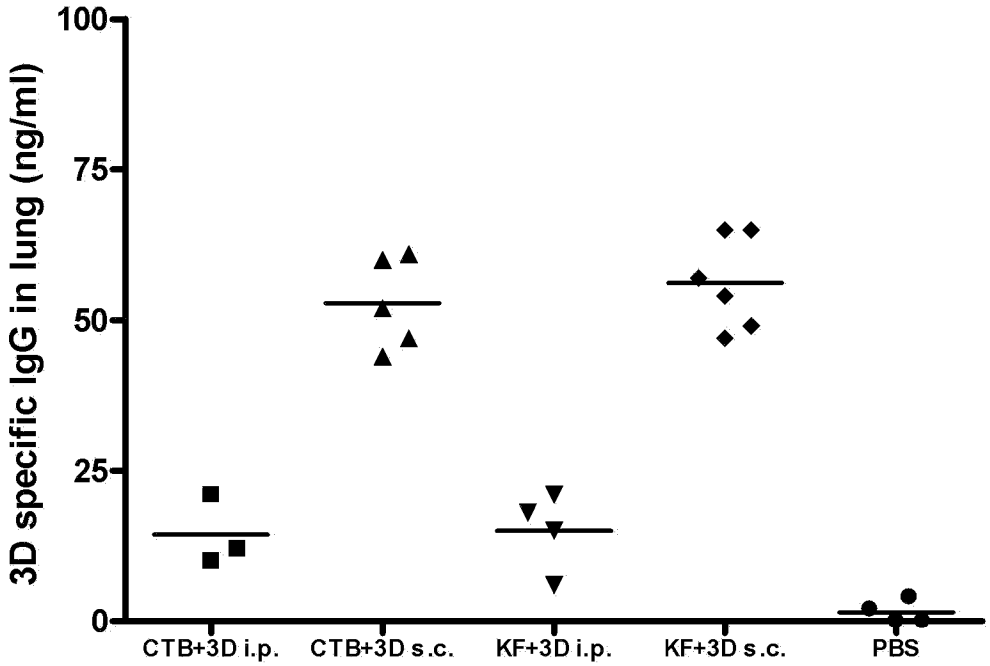

100
80
60
40
20
0
Percent survival
0
1
2
3
4
5
6
7
8
Days post of challenge
PBS
EV71
VTT-3D
3D

FIG 13

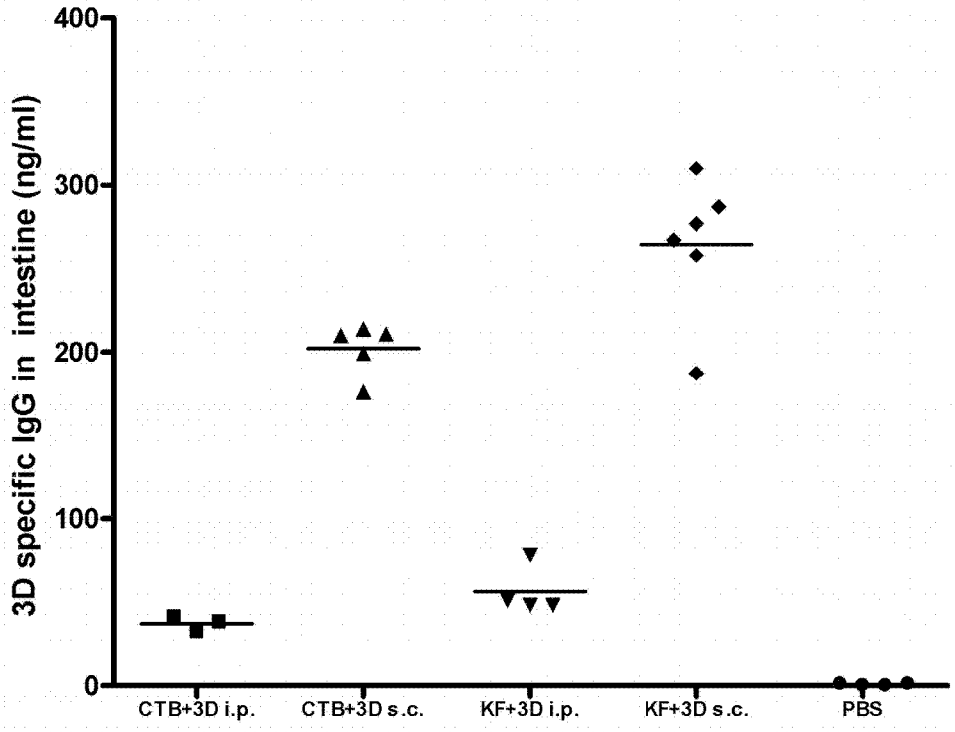

3D-specific IgG in serum (ug/ml)
0
10
20
30
40
50
CTB+3D i.p.
CTB+3D s.c.
KF+3D i.p.
KF+3D s.c.
PBS

(a)

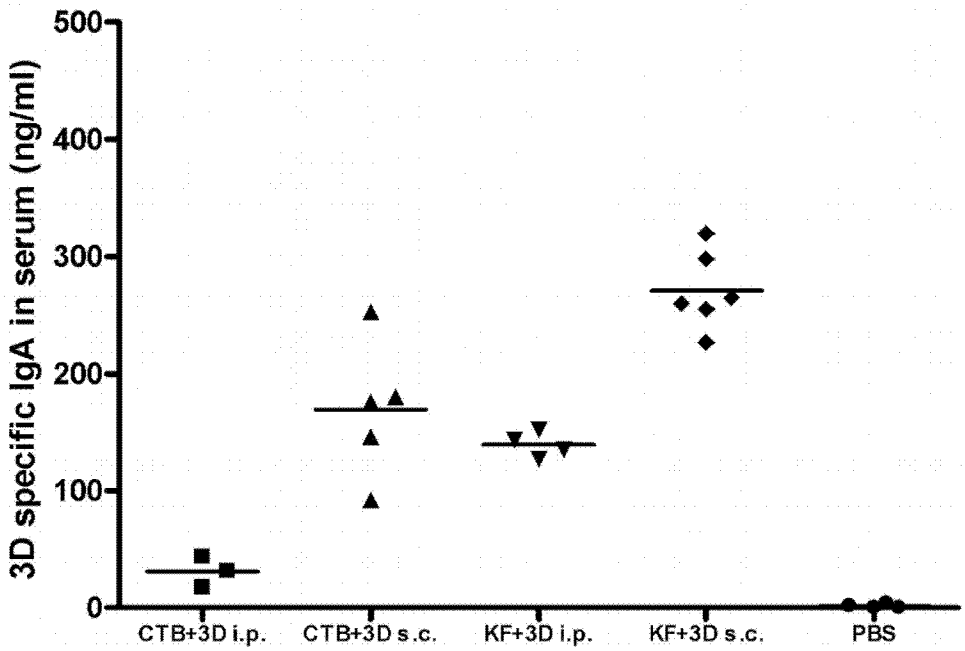

3D specific IgG in lung (ng/ml)
0
25
50
75
100
CTB+3D i.p.
CTB+3D s.c.
KF+3D i.p.
KF+3D s.c.
PBS

(b)

FIG 14

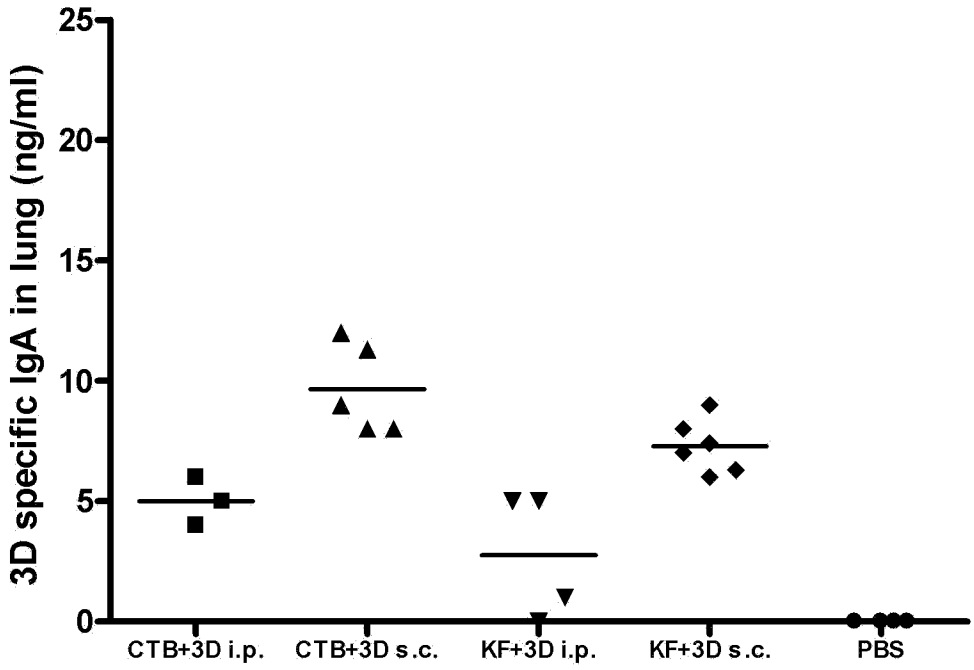



(c)

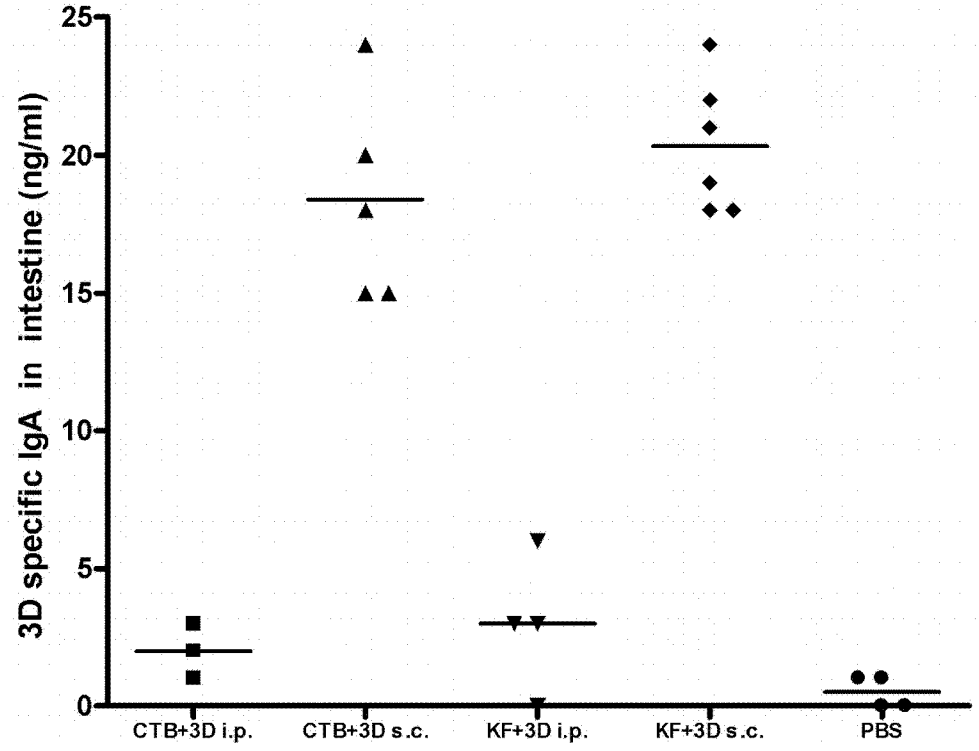



(d)

FIG 14 (Cont'd)




(e)




(f)

FIG 14 (Cont'd)

# INTERNATIONAL SEARCH REPORT

International application No.
**PCT/CN2015/071022**

## A. CLASSIFICATION OF SUBJECT MATTER

C07K 16/10(2006.01)i; A61K 39/125(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K; A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CNABS;DWPI;CNKI;ISI Web of knowledge and keywords:3d protein, enterovirus, antigen?, antibod??? EMBL,GenBank and amino acid sequences: SEQ ID NOs:1-8,10-14

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | CN 101699291 A (INST OF LAB ANIMAL SCIENCES CA) 28 April 2010 (2010-04-28)<br>See the entire document | 1-9 |
| A | WO 2008070924 A1 (PICORAL PTY LTD) 19 June 2008 (2008-06-19)<br>See the entire document | 1-9 |
| X | Kiener T.K. et al. "Characterization of a monoclonal antibody against the 3D polymerase of enterovirus 71 and its use for the detection of human enterovirus A infection"<br>*Journal of Virological Methods,* Vol. Volume 180, No. Issues 1-2,<br>31 December 2011 (2011-12-31),<br>ISSN: ISSN: 0166-0934,<br>See the entire document, especially the abstract and page 76, right column, Section 2.3 | 5-6 |
| A | Kiener T.K. et al. "Characterization of a monoclonal antibody against the 3D polymerase of enterovirus 71 and its use for the detection of human enterovirus A infection"<br>*Journal of Virological Methods,* Vol. Volume 180, No. Issues 1-2,<br>31 December 2011 (2011-12-31),<br>ISSN: ISSN: 0166-0934,<br>See the entire document, especially the abstract and page 76, right column, Section 2.3 | 1-4, 7-9 |

☑ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

* Special categories of cited documents:

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| **22 September 2015** | **10 October 2015** |

| Name and mailing address of the ISA/CN | Authorized officer |
|---|---|
| **STATE INTELLECTUAL PROPERTY OFFICE OF THE P.R.CHINA**<br>**6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing 100088, China** | **JIA,Tao** |
| Facsimile No. **(86-10)62019451** | Telephone No. **(86-10)62411993** |

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | Dahllund L. et al. "Immunological reactivity of baculovirus-expressed enterovirus proteins" *JOURNAL OF VIROLOGICAL METHODS*, Vol. Volume 67, No. Number 2, 30 September 1997 (1997-09-30), ISSN: ISSN:0166-0934, See the entire document | 1-9 |
| A | AUVINEN P. et al. "Mapping of antigenic sites of coxsackievirus B3 by synthetic peptides" *APMIS*, Vol. Volume 101, No. Issue 7-12, 31 July 1993 (1993-07-31), ISSN: ISSN:1600-0463, See the entire document | 1-9 |

**INTERNATIONAL SEARCH REPORT**
**Information on patent family members**

International application No.
**PCT/CN2015/071022**

| Patent document cited in search report | Publication date (day/month/year) | Patent family member(s) | Publication date (day/month/year) |
|---|---|---|---|
| CN 101699291 A | 28 April 2010 | None | |
| WO 2008070924 A1 | 19 June 2008 | US 2010196874 A1 | 05 August 2010 |
| | | AU 2007332082 A1 | 19 June 2008 |