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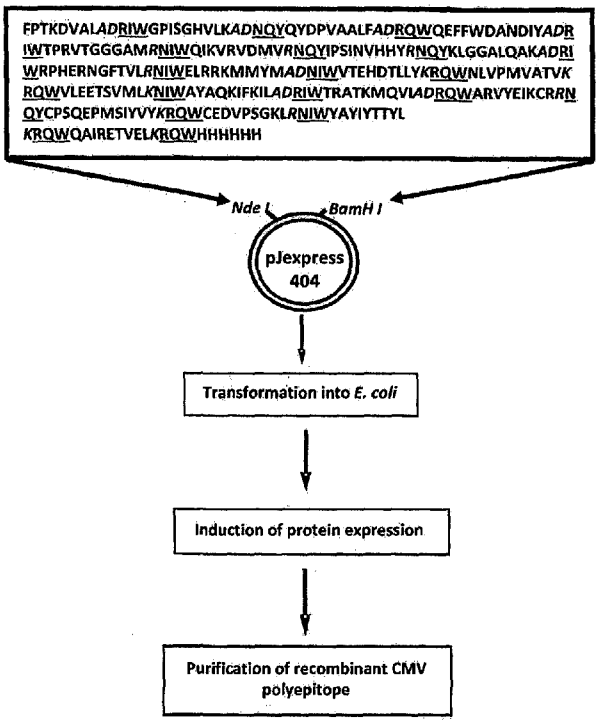

Figure 1

(57) Abstract: An isolated protein comprises respective amino acid sequences of each of a plurality of CTL epitopes from two or more different herpesvirus antigens and further comprises an intervening amino acid or amino acid sequence between at least two of said CTL epitopes comprising proteasome liberation amino acids or amino acid sequences and, optionally, Transporter Associated with Antigen Processing recognition motifs. The isolated protein is capable of rapidly expanding human cytotoxic T lymphocytes (CTL) in vitro and eliciting a CTL immune response in vivo upon administration to an animal as an exogenous protein. Typically, the isolated protein comprises no more than twenty (20) CTL epitopes derived from cytomegalovirus and/or Epstein-Barr virus antigens.

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TITLE

IMPROVED HUMAN HERPESVIRUS IMMUNOTHERAPY

TECHNICAL FIELD

THIS INVENTION relates to human herpesvirus immunotherapy. In particular, the invention relates to a recombinant protein which includes a plurality of cytotoxic T cell epitopes derived from multiple human cytomegalovirus (CMV) or Epstein-Barr virus (EBV) antigens, which, when used in immunotherapy are capable of eliciting a cytotoxic T-lymphocyte immune response, without being limited thereto.

BACKGROUND

Epstein-Barr virus occurs with an extremely high incidence with over 90% of adults showing some sign of exposure. EBV also persists subsists as a lifelong latent infection and may be asymptomatic. However, EBV can result in mononucleosis, also known as glandular fever causing significant morbidity in some individuals. EBV may be associated with several autoimmune diseases such as lupus, rheumatoid arthritis and multiple sclerosis. Importantly, EBV is known to be associated with a number of cancers such as nasopharyngeal carcinoma (NPC), Burkitt's lymphoma and Hodgkin lymphoma. NPC is a cancer that is common in Chinese and South-East Asian populations (rare in most other populations). Patients often present with mid (Stage III) or advanced stage (Stage IV) disease as symptoms are poorly recognised at earlier stages. The first line of treatment for patients when diagnosed with NPC is radiotherapy and chemotherapy with limited options for surgery. Radio/chemo is effective for many patients but approximately 20% will either respond inadequately or relapse and this group have a poor prognosis. Patients that present with stage III and IV tumours have a 5 year overall survival of only 50 to 60% (lower for stage IV patients alone). The most common forms of NPC are associated with EBV making these tumours amendable to immunotherapy by targeting and killing EBV infected tumour cells.

Primary CMV in healthy individuals is generally asymptomatic, establishing a latent state with occasional reactivation and shedding from mucosal surfaces. In some cases primary CMV infection is accompanied with clinical symptoms of a mononucleosis-like illness, similar to that caused by Epstein-Barr virus. There are two important clinical settings where CMV causes significant morbidity and mortality. These include congenital primary infection and primary or reactivation of virus in immunosuppressed adults. In the

congenital setting, CMV is the leading cause of mental retardation and other abnormalities such as deafness in children and this impact has been emphasized by its categorization by the Institute of Medicine as a Level I vaccine candidate [i.e. most favourable impact–saves both money and quality-adjusted life years (QALYs) (Arvin, Fast et al. 2004). CMV-associated complications in immunocompromised individuals such as HIV-infected individuals is often seen in patients with $CD4^+$ T cell counts below 50/μl (Palella, Delaney et al. 1998; Salmon-Ceron, Mazeron et al. 2000). In addition, the impact of CMV in transplant patients, including both solid organ transplant and allogeneic hematopoietic stem cell transplant recipients, is well recognized.

Primary exposure to CMV results in the induction of a strong primary immune response, which is maintained as a long-term memory response, and serves to restrict viral replication following reactivation. There is now firm evidence that both humoral and cellular immune responses play a crucial role in controlling CMV infection. Studies carried out in murine CMV models provided the initial evidence on the importance of T cell immunity, where a loss of T cell function was co-incident with increased reactivation and dissemination of viral infection (Reddehase, Weiland et al. 1985; Mutter, Reddehase et al. 1988). Furthermore, the reconstitution of virus-specific T cell immunity was coincident with recovery from acute viral infection. Subsequent studies in humans under different clinical settings have further emphasized the role of virus-specific T cells. These studies showed that allogeneic stem cell transplant patients, who had insufficient anti-viral T cell immunity, demonstrated an increased risk of developing CMV-associated complications. Convincing evidence for the role of cellular immunity in the control of CMV-disease came from studies where adoptive transfer of donor derived CMV-specific $CD8^+$ T cells not only restored antigen-specific cellular immunity, but also prevented CMV-associated clinical complications in allogeneic stem cell transplant patients (Riddell, Watanabe et al. 1992; Walter, Greenberg et al. 1995).

Taking these studies into consideration, a variety of CMV vaccines have been evaluated in preclinical and clinical trials.

These CMV vaccine strategies have assessed glycoprotein B (gB), pp65 and IE-1 as potential targets and they have been delivered by numerous delivery platforms, including the attenuated CMV Towne strain (Jacobson, Sinclair et al. 2006), recombinant viral vectors encoding full length antigens and epitopes (Bernstein, Reap et al. 2009; Zhong and Khanna 2009), DNA (Wloch, Smith et al. 2008), dense body (Frankenberg, Pepperl-

Klindworth et al. 2002), and subunit (Drulak, Malinoski et al. 2000) vaccines. However, none of these approaches have shown convincing clinical efficacy and have not entered into clinical practice.

Typically, it has been proposed that in order to elicit a protective, $CD8^+$ cytotoxic T cell response, viral antigens must be delivered in nucleic acid form (*e.g* using a viral vector delivery system) rather than as an exogenously-delivered proteins so that the expressed protein is properly processed and presented to T cells (Koup & Douek, 2012). The majority of these vaccine delivery platforms, in particular live-attenuated vaccines and viral vector based vaccines, have raised several regulatory concerns such as perceived long-term theoretical health risks (Liu ; Soderberg-Naucler 2006; Anderson and Schneider 2007).

SUMMARY

The present invention addresses a need for the development of herpesvirus immunotherapy using a safe delivery technology. The invention is directed towards reducing the risk of CMV associated injury to the developing fetus, and immunologically compromised individuals such as recipients of solid organ and hematopoietic stem cell transplants and patients with advanced HIV disease. The invention is also directed toward treating the symptoms of an existing EBV infection, such as in immunologically compromised transplant patients or in the prevention or treatment of EBV-associated cancers such as nasopharyngeal carcinoma (NPC).

The invention has surprisingly arisen from the discovery that contrary to past assumptions, an exogenous a polyepitope protein administered to an individual may elicit a protective, $CD8^+$ cytolytic T cell response.

Accordingly, the invention is broadly directed to an isolated polyepitope protein comprising a plurality of human herpesvirus cytotoxic T cell (CTL) epitopes that is capable of eliciting a cytotoxic T cell response.

In a first aspect, an isolated protein comprises respective amino acid sequences of each of a plurality of CTL epitopes from two or more different herpesvirus antigens and which further comprises an intervening amino acid or amino acid sequence between at least two of said CTL epitopes comprising proteasome liberation amino acids or amino acid sequences and, optionally, Transporter Associated with Antigen Processing (TAP) recognition motifs, wherein the isolated protein is capable of eliciting a cytotoxic T-lymphocyte immune response upon administration to an animal as an exogenous protein.

Suitably, the isolated protein comprises epitopes are selected to provide broad coverage of the human population. These include HLA class I specificities HLA-Al, -A2, -A3, -All, -A23, -A24, -A26, -A29, - A30, -B7, -B8, -B27, - B35, -B38, -B40, -B41, -B44, -B51, -B57 and -B58.

Suitably, said plurality of epitopes comprises less than twenty (20) epitopes in total.

In one embodiment, the herpesvirus is CMV. Preferably, the CTL epitopes are from CMV antigens selected from the group consisting of: pp50, pp65, pp150 and IE-l.

In a preferred embodiment, the isolated protein comprises a plurality of CTL epitopes selected from Table 1 (SEQ ID NOS: 1-21) In a particular embodiment, the isolated protein comprises a plurality of CTL epitopes selected from Table 2 (SEQ ID NOS: 1-13).

In a preferred embodiment, at least one of the CTL epitopes comprises the amino acid sequence VTEHDTLLY (SEQ ID NO:11).

In another embodiment, the herpesvirus is EBV.

Preferably, the CTL epitopes are from EBV antigens selected from the group consisting of: BMLF1, LMP2a, BRLF1, LMP2, EBNA3A, BZLF1, EBNA3C, EBNA1 andEBNA3B.

In a preferred embodiment, the isolated protein comprises a plurality of CTL epitopes selected from and Table 3 (SEQ ID NOS:22-41).

It will also be appreciated that the isolated protein may comprise CTL epitopes from the same or different herpesvirus (*e.g* CMV and/or EBV).

The isolated protein may further comprise intervening amino acids or amino acid sequences.

In a preferred embodiment, the intervening amino acids or amino acid sequences are proteasome liberation amino acids or amino acid sequences.

In an optional embodiment, the intervening amino acids or amino acid sequence are Transporter Associated with Antigen Processing (TAP) recognition motifs.

In a second aspect, the invention provides an isolated nucleic acid encoding the isolated protein of the first aspect.

In a third aspect, the invention provides a genetic construct comprising the isolated nucleic acid of the second aspect.

Preferably, the genetic construct is an expression construct wherein said isolated

nucleic acid of the second aspect is operably linked to one or more regulatory sequences present in an expression vector.

In an embodiment, the expression construct comprises an expression vector suitable for production of the isolated protein *in vitro* as a recombinant protein for subsequent purification.

In a fourth aspect, the invention provides a host cell comprising the expression construct of the third aspect.

In another embodiment, the host cell has been transfected, transformed or otherwise introduced with the expression construct *in vitro*, for the purpose of subsequent purification of the isolated protein of the first aspect.

In a fifth aspect, the invention provides a method of producing the isolated protein of the first aspect, said method including the steps of expressing the isolated protein in the host cell of the fourth aspect and at least partly purifying the isolated proteins under conditions that maintain the isolated protein in a substantially non-aggregated form.

In a sixth aspect, the invention provides an isolated protein produced according to the method of the fifth aspect.

In a seventh aspect, the present invention provides a pharmaceutical composition comprising the isolated protein of the first or sixth aspects or the genetic construct of the third aspect, and a pharmaceutically-acceptable carrier, diluent or excipient.

Preferably, the pharmaceutical composition is an immunogenic composition suitable for use in the prophylactic or therapeutic treatment of a disease or condition associated with CMV and/or EBV infection in an animal.

More preferably, the immunotherapeutic composition is a vaccine for eliciting a protective immune response against CMV and/or EBV. In this regard, it will be appreciated that the pharmaceutical composition may comprise separate isolated proteins respectively comprising CMV and EBV CTL epitopes or may comprise a single isolated protein comprising both EBV and CMV epitopes.

In one particular embodiment, the pharmaceutical composition further comprises one or more immunostimulatory molecules or adjuvants.

Suitably, the immunostimulatory molecule or adjuvant comprises one or more toll-like receptor (TLR) agonists.

Preferably, the TLR agonists include a TLR4 agonist and/or a TLR9 agonist.

Preferred adjuvants include Monophosphoryl lipid (MPL) and/or immunostimulatory DNA such as CpG ODN1826, CpG ODN2006, CpG ODN2216 and/or CpG ODN2336, although without limitation thereto.

In an eighth aspect, the invention provides a method of prophylactically or therapeutically treating a herpesvirus infection in an animal including the step of administering to the animal the isolated protein of the first or sixth aspects, or the pharmaceutical composition of the seventh aspect, to thereby prophylactically or therapeutically treat the herpesvirus infection in the animal.

In particular embodiments, the herpesvirus is CMV or EBV.

In a ninth aspect, the invention provides a method of inducing a cytotoxic T-lymphocyte (CTL) immune response in an animal including the step of administering to the animal the isolated protein of the first or sixth aspects or the pharmaceutical composition of the seventh aspect, to thereby induce or elicit a cytotoxic T-lymphocyte (CTL) immune response in said animal.

In a tenth aspect, the invention provides a method of expanding herpesvirus-specific CTLs for adoptive immunotherapy, including the steps of:

(i) contacting one or more cells isolated from an animal with the isolated protein of the first or sixth aspects; and

(ii) culturing said one or more cells to thereby expand herpesvirus -specific CTLs from said one or more cells.

In particular embodiments, the herpesvirus is CMV or EBV.

In an eleventh aspect, the invention provides a method of adoptive immunotherapy including the step of administering said herpesvirus -specific CTLs produced at step (ii) of the tenth aspect to an animal to thereby prophylactically or therapeutically treat a herpesvirus infection of said animal.

In particular embodiments, the herpesvirus is CMV or EBV.

In a twelfth aspect, the invention provides the isolated protein of the first or sixth aspects, or the genetic construct of the third aspect for use in prophylactically or therapeutically treating a herpesvirus infection in an animal.

In particular embodiments, the herpesvirus is CMV or EBV.

Preferably, according to the aforementioned aspects the animal is a mammal. More preferably, the animal is a human.

BRIEF DESCRIPTION OF THE FIGURES

In order that the present invention may be more readily understood and placed into practical effect, preferred embodiments of the invention will be described, by way of example only, with reference to the accompanying figures.

Figure 1: Illustration of the design of the CMV polyepitope and downstream processing. The design of the CMV polyepitope 20mer encoding sequence is shown as an example. Individual epitope amino acid sequences are shown in bold; grey, Italicised letters following the epitope sequence represent the amino acid residues for processing of the CMV polyepitope protein by the proteasome and the underlined amino acid sequences represent the motifs for TAP (referred to as CMVpoly-PTL). The DNA sequence encoding the CMV polyepitope protein was synthetically made, cloned into an E.coli inducible plasmid, pJexpress 404, and transformed into E. coli to carry out protein expression and purification.

Figure 2: Expression and purification of CMVpoly-PTL proteins. The pJexpress 404 plasmids expressing the CMVpoly-PTL proteins which include 13, 14, 15 or 20 CMV $CD8^+$ T cell epitopes were transformed into E.coli BL21 (DE3) pLysS. Protein expression was induced with IPTG and pre and post induction samples were analysed using SDS PAGE. Panel A and B shows expression of CMVpoly-PTL proteins in E. Coli: Lane 1, molecular weight marker (kDa); Lanes 2, 4 and 6 uninduced E. coli cell lysate; Lanes 3, 5 & 7 induced E. coli cell lysate. * indicates the CMVpoly-PTL proteins.

Figure 3: SDS PAGE analysis of purified CMVpoly-PTL proteins Following CMVpoly-PTL purification on Ni NTA column, samples from various stages of purification were analysed by SDSPAGE. Panels A, B, C & D represent the purification of the CMVpoly-PTL proteins (13mer, 14mer, 15mer and 20mer). For all the SDS PAGE gels Lane 1: molecular weight marker. Lane 2: solubilised protein prior to loading. Lane 3: flow through. Lane 4: wash. Lanes 5, 6, 7 & 8: elution fractions. * indicates CMVpoly-PTL proteins.

Figure 4: CMVpoly-PTL protein solubility test and characterisation to determine a compatible buffer system for CMVpoly-PTL storage as a soluble protein, purified protein was diluted with various buffer compositions at different pH ranges, incubated at 4^0C O/N,

centrifuged and supernatant fractions were analysed on SDS PAGE. Panel A: Lane 1: molecular weight marker. Lane 2: diluted with 25 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer pH 5.6. Lane 3: diluted with 25 mM MES buffer pH 3.2. Lane 4: diluted with 25 mM MES pH 4.5. Lane 5: diluted with 25 mM MES pH 4.5 and 400 mM L arginine. Lane 6: diluted with 10 mM Tris and 100 mM NaH_2Po_4 pH 4.3. Lane 7: diluted with 10 mM Tris, 100 mM NaH_2Po_4 and 400 mM L arginine pH 4.3. Lane 8: diluted with PBS, 50 mM L-arginine and 50 mM L-glutamic acid pH7.4. Lane 9: diluted with water. Lane 10: diluted with 100 mM glycine buffer pH 2. Panel B, C & D shows CMVpoly-PTL proteins purity analysis. Following dialysis of the CMVpoly-PTL polyepitope proteins (13mer, 14mer and 15mer) against MES buffer pH 5.6, different concentrations of each protein was analysed on SDS PAGE to observe the final purity and degradation products.

Figure 5: Expansion of CMV-specific T cells following stimulation of PBMCs from CMV seropositive donors with the CMVpoly-PTL proteins: PBMC from various healthy CMV-seropositive donors were stimulated *ex vivo* with recombinant CMVpoly-PTL protein (13, 14 and 15mer) and cultured for 10 days in the presence of recombinant IL 2. The percentage of expanded peptide-specific $CD8^+$ T cells producing IFN-γ was determined using an ICS assay and results were analysed using FlowJo. Panel A shows the representative FACS plots of in vitro expanded CMV-specific $CD8^+$ T cells following stimulation of PBMC with or without the CMVpoly-PTL proteins. Panel B & C shows overall analysis of expanded CMV specific $CD8^+$ T cells from different individuals following stimulation with CMVpoly-PTL proteins (13, 14 and 15mer).

Figure 6: The magnitude and quality of expanded CMV specific $CD8^+$ T cells following stimulation with CMV polyepitope protein: Following PBMC stimulation with the CMVpoly-PTL protein (13mer), cells were analysed to assess for effector functions by multi parameter flow cytometry. The frequency of $CD8^+$ T cells demonstrating cytolytic function (CD107a degranulation marker) and intracellular cytokine production (IFN γ, TNF and MIP 1β) were analysed on FlowJo and multifunctional cytokine producers were plotted using the SPICE program. Data in the pie chart is shown for an individual epitope and each slice of the pie chart represents each possible combination of functions.

Figure 7: Schematic design of the CMV polyepitope protein construct with and without linkers and protein purification: Panel A shows the design of CMV polyepitope protein without linkers (SEQ ID NO:46) (referred to as CMVpoly). Panel B shows the design of polyepitope protein with proteasome linkers (SEQ ID NO: 47) (referred to as

CMVpoly-PL). Each of the alternate $CD8^+$ T cell epitope sequences are italicised and underlined. For CMVpoly-PL each epitope sequence is separated by amino acid residue(s) which are targets for proteasomal degradation (shown in red). The DNA sequence encoding the CMV polyepitope proteins was cloned into an IPTG inducible plasmid, pJexpress 404, and transformed into E. coli for protein expression. Polyepitope protein was purified using Ni-NTA affinity chromatography.

Figure 8: In vitro assessment of processing and presentation of CMVpolyepitope proteins with and without linkers: Panel A shows in vitro cross-presentation of CMVpoly, CMVpoly-PL and CMVpoly-PTL proteins by human cells. EBV transformed LCLs were pulsed with CMVpoly, CMVpoly-PL or CMVpoly-PTL proteins (25 μg each) for two hours, washed, incubated overnight and then exposed to CMV-specific $CD8^+$ T cells specific for HLA A2-restricted NLV (pp65), HLA A1-restricted VTE (pp50), HLA B8-restricted ELR (IE1), HLA B7-restricted RPH (pp65) and HLA B7-restricted TPR (pp65) epitopes. The FACS plots shows IFN-γ expression by the CMV-specific $CD8^+$ T cells following co-culture with CMVpoly, CMVpolyPL or CMVpoly-PTL proteins pulsed LCLs. Panel B shows the mean ± SEM of IFN-γ producing CMV epitope specific $CD8^+$ T cells following co-culture with LCL pulsed with CMVpoly (empty bars), CMVpoly-PL (black bars) or CMVpoly-PTL (grey bars). Error represent the ± SEM. ** or *** indicates statistically significant ($p<0.001$ or $p<0.0001$), calculated by 2-tailed Student's t test.

Figure 9: Analysis of the cross-presentation of the CMV polyepitope protein by human cells: To identify the role of peptide transporters (TAP-1 and TAP-2) in the cross-presentation of CMV polyepitope protein, TAP1&2+ cells (CEM.T1) and TAP1&2- cells (CEM.T2 or CEM.T2 HLA B7) were pulsed with CMV-PTL protein for two hours, washed, incubated overnight and exposed to HLA A2-restricted NLV (pp65) or HLA B7-restricted TPR (pp65) epitope-specific CD8+ T cells. Panel A shows expression of IFN γ by NLV-specific T cells following exposure of CEM.T1 cells pre-sensitized with CMV polyepitope protein. Panel B & C shows the percentage of IFN-γ expressing NLV and TPR-specific $CD8^+$ T cells following exposure to CMV polyepitope protein sensitized CEM.T2 and CEM.T2 HLA B7 cells respectively. The data shown in panels A, B & C is one representative experiment from two independent experiments.

Figure 10: The effect of different chemical inhibitors on the processing and presentation of the polyepitope protein: CEM.T1 and CEM.T2 cells were either untreated or pre treated with inhibitors for autophagy (3- MA), lysosomes/endosome (chloroquine or bafilomycin

A1), the recycling pathway (primaquine), cysteine proteases (leupeptin or E64) or acid proteases (pepstatin A) (Panel A), proteasomal inhibitors, lactacystin, epoxomicin and MG132 (Panel B) and ER-resident aminopeptidase inhibitor (leucinethiol + DTT) or its control (DDT alone) or golgi inhibitors (brefeldin A or monensin) (Panel C) prior to incubation with the CMV-PTL protein. Cells were washed and cultured in the presence of respective inhibitors for twelve hours and then exposed to HLA A2-restricted NLV (pp65)-specific CD8+ T cells and then assessed for IFN-γ expression by ICS assay. Data presented in each represents the relative IFN-γ expression by antigen-specific T cells following exposure to CMV-PTL sensitized CEM.T1 (empty bars; referred to as T1) and CEM.T2 (black bars; referred to as T2) cells. The data represents the mean of two independent experiments performed in triplicates. Error bars represent the ± SEM. * or** indicates statistically significant ($p<0.05$ or $p<0.01$), calculated by 2 tailed Student's test.

Figure 11: Effect of Sec61 and ATG12 shRNA on the cross-presentation of the polyepitope protein: CEM T1 and CEM T2 cells were transduced with recombinant lentivirus encoding shRNA for Sec61β subunit or ATG12 or a control vector (pLKO), cultured for two days in R 10 medium, selected in puromycin for seven days and then used as antigen presenting cells. Panel A & D shows western blot analysis of Sec61 and ATG12 protein expression in CEM.T1 and CEM.T2 cells following transduction of shRNA. GAPDH was used as a control for protein loading. Panels B-F shows the expression of IFN-γ by CMV-specific $CD8^+$ T cells following exposure to CMVpoly-PTL sensitized CEM.T1 and CEM.T2 cells transduced with Sec61 and ATG12 shRNA lentivirus or control vector.

Figure 12: In vivo assessment of immunogenicity of CMVpoly, CMVpoly-PL and CMVpoly-PTL proteins: To assess the immunogenicity of CMVpoly, CMVpoly-PL or CMVpoly-PTL proteins, 20μg of protein was formulated with 25μg of MPL (monophosphoryl lipid A) and 50μg of CpG ODN1826 in 100μL volume per dose. On day 0, 6-8 weeks old HLA A2 transgenic mice were immunised subcutaneously and a booster dose was give with an identical formulation on day 21. Mice were sacrificed on day 35, splenocytes were stimulated in vitro with HLA A2-restricted NLV (pp65) and HLA A2-restricted VLE (IE-1) peptide epitopes for 10 days in the presence of IL-2 and then assessed for cytokine expression using ICS assays. Panel A shows the frequencies of CMV-specific $CD8^+$ T cells following immunisation with CMVpoly, CMVpoly-PL or CMVpoly-PTL-based vaccine formulation. Panel B shows the absolute percentage of CMV-specific $CD8^+$ T cells expressing different combination of cytokines (IFN- γ, TNF and/or IL-2) following

vaccination with CMVpoly, CMVpoly-PL or CMVpoly-PTL proteins. Error bars represent the mean ± SEM. * indicates statistically significant ($p<0.05$).

Figure 13: Schematic design of the Epstein-Barr virus (EBV) polyepitope construct with proteasome linkers and protein purification. Panel A shows the design of EBV polyepitope protein with proteasome linkers (referred to as EBVpoly). Each of the alternate $CD8^+$ T cell epitope sequences are italicised and underlined. For EBVpoly each epitope sequence is separated by amino acid residue(s), which are targets for proteasomal degradation (shown in red). Panel B shows the purification of EBVpoly protein. The DNA sequence encoding the EBVpoly protein was cloned into an IPTG inducible plasmid, pJexpress 404, and transformed into E. coli for protein expression. EBVpoly protein was purified using Ni-NTA affinity chromatography and then analysed using SDS-PAGE. Predicted size for the EBVpoly was 25Kd.

Figure 14: In vitro expansion of EBV-specific $CD8^+$ T cells from healthy sero-positive donors using EBVpoly protein. PBMC from a panel of healthy donors (n=8) were stimulated with or without EBVpoly protein in vitro, cultured for 14 days in the presence of IL-2 and then cells were assessed for the expansion of EBV-specific T cells using ICS assays. Bar graphs represent the comparative percentage of expanded EBV-specific $CD8^+$ T cells from each donor following stimulation with EBVpoly protein.

Fig. 15: Amino acid sequences of CMV and EBV polyepitope proteins and nucleotide sequences of encoding nucleic acids. A: CMV polytope is SEQ ID NO:42; nucleotide sequence encoding CMV polytope is SEQ ID NO:50; B: CMV polytope is SEQ ID NO:43; nucleotide sequence encoding CMV polytope is SEQ ID NO:51; C: CMV polytope is SEQ ID NO:44; nucleotide sequence encoding CMV polytope is SEQ ID NO:52; D: CMV polytope is SEQ ID NO:45; nucleotide sequence encoding CMV polytope is SEQ ID NO:53; E: CMV polytope is SEQ ID NO:46; nucleotide sequence encoding CMV polytope is SEQ ID NO:54; F: CMV polytope is SEQ ID NO:47; nucleotide sequence encoding CMV polytope is SEQ ID NO:55; G: CMV polytope is SEQ ID NO:48; nucleotide sequence encoding CMV polytope is SEQ ID NO:56; H: EBV polytope is SEQ ID NO:49; nucleotide sequence encoding EBV polytope is SEQ ID NO:57.

DETAILED DESCRIPTION

The present invention is at least partly predicated on the unexpected discovery that an isolated protein comprising a plurality of herspesvirus epitopes such as CMV and/or EBV epitopes administered to an individual as an exogenous protein may elicit a protective,

$CD8^+$ cytotoxic T cell response. It appears that once administered, the exogenous protein is processed by a novel, cellular TAP-independent, proteasome and autophagy dependent pathway which is assisted by the inclusion of proteasome liberation amino acids in the exogenous protein. This results in HLA Class I-dependent presentation of the processed CMVepitopes to $CD8^+$ cytotoxic T cells. This unexpected discovery may also be at least partly related to an improved recombinant protein purification method that avoids or reduces aggregation of the recombinant protein. A difficulty typically encountered with such proteins is that T cell epitopes are hydrophobic and/or contain several hydrophobic amino acids, which means that the protein is susceptible to hydrophobic aggregation, which may compromise the ability to deliver the recombinant protein in a manner which enables the CTL epitopes of the protein to be processed in the manner described above. This is exacerbated by the use of intervening TAP recognition motifs that are typically hydrophobic. The improved recombinant polyepitope protein purification method described herein avoids or at least reduces aggregation of the polytope protein, thereby allowing efficient delivery and processing of the polyepitope protein. The inventors have also discovered that production, purification and immunization with the isolated polyepitope protein is optimized by using less than twenty (20) CTL epitopes in the isolated protein. Further to the above, the invention utilizes particular immunogenic components such as toll-like receptor (TLR) agonists that enhance the immunogenicity of the isolated protein.

Throughout this specification, unless otherwise indicated, "*comprise*", "*comprises*" and "*comprising*" are used inclusively rather than exclusively, so that a stated integer or group of integers may include one or more other non-stated integers or groups of integers.

It will also be appreciated that the indefinite articles "*a*" and "*an*" are not to be read as singular indefinite articles or as otherwise excluding more than one or more than a single subject to which the indefinite article refers. For example, "a" protein includes one protein, one or more proteins or a plurality of proteins.

In a first aspect, an isolated protein comprises respective amino acid sequences of each of a plurality of CTL epitopes from two or more different herpesvirus antigens and which further comprises an intervening amino acid or amino acid sequence between at least two of said CTL epitopes comprising proteasome liberation amino acids or amino acid sequences and, optionally, Transporter Associated with Antigen Processing (TAP) recognition motifs,wherein the isolated protein is capable of eliciting a cytotoxic T-lymphocyte immune response upon administration to an animal as an exogenous protein.

By *"isolated"* is meant material that has been removed from its natural state or otherwise been subjected to human manipulation. Isolated material may be substantially or essentially free from components that normally accompany it in its natural state, or may be manipulated so as to be in an artificial state together with components that normally accompany it in its natural state.

By *"protein"* is meant an amino acid polymer comprising natural and/or non-natural amino acids, D- or L- amino acids as are well known in the art.

A *"peptide"* is a protein having no more than fifty (50) amino acids.

A "*polypeptide*" is a protein having more than fifty (50) amino acids.

As used herein, the isolated protein may be referred to as an isolated polyepitope or polytope protein. For example, an isolated "*CMV polyepitope*", "*EBV polyepitope*" or an isolated "*CMV polyepitope protein*" or "*EBV polyepitope protein*".

In the context of the present invention, an "*exogenous*" protein or polyepitope protein is a protein produced externally to the animal to which it is subsequently administered. Effectively, the exogenous protein is administered or administrable to the animal, rather than being produced or expressed by the animal *in situ* (*e.g.* by cells or tissues of the animal) following delivery of a nucleic acid or genetic construct encoding the protein to the animal. A preferred exogenous protein is a recombinant protein produced in an isolated host cell ex *vivo*, such as a bacterial host cell.

As used herein, a "*CTL epitope*" is a peptide, or an amino acid sequence of the peptide, that is capable of stimulating or activating a cytotoxic T lymphocyte to recognize a target cell presenting the epitope in the context of the appropriate MHC Class I molecule. Recognition of the target cell may include or result in cytokine production (e.g,. IFN-γ, IL-2, MIP-1β and/or TNF), changes in cell surface marker expression (*e.g.* CD107a) and/or lysis and/or killing of the target cell.

Typically, although not exclusively, a CTL epitope comprises 7, 8, 9, 10, 11, 12, 13, 14 or 15 contiguous amino acids of, derived from, obtained from or based on a corresponding herpesvirus antigen.

The polyepitope protein preferably comprises a plurality of CMV and/or EBV CTL epitopes derived from a plurality of different CMV protein antigens. Preferably, the epitopes are of CMV antigens selected from the group consisting of: pp50, pp65 , pp150 and IE-l and/or EBV antigens selected from the group consisting of: BMLF1, LMP2a, BRLF1, LMP2, EBNA3A, BZLF1, EBNA3C, EBNA1 andEBNA3B.

Suitably, the CMV and/or EBV polyepitope protein comprises CTL epitopes selected to provide broad coverage of a population. In humans, these include HLA class I specificities HLA-Al, -A2, -A3, -All, -A23, -A24, -A26, -A29, - A30, -B7, -B8, -B27, -B35, -B38, -B40, -B41, -B44, -B51, -B57, -B58 and –cw6.

In certain embodiments, the CTL epitopes are restricted to the HLA class I specificities shown in Table 1 or Table 2.

In a particular embodiment, the CMV polyepitope protein comprises a plurality of HLA class I restricted CTL epitopes selected from Table 1 (SEQ ID NOS: 1-21) or Table 3 (SEQ ID NOs: 22-41).

In a particular embodiment, the EBV polyepitope protein comprises a plurality of HLA class I restricted CTL epitopes selected from Table 3 (SEQ ID NOs: 22-41).

It will also be appreciated that the invention contemplates inclusion of CTL epitopes derived from the same or different herpesvirus (*e.g* CMV and/or EBV). Accordingly, one embodiment of the isolated protein comprises CTL epitopes from both CMV and EBV antigens.

Suitably, said plurality of epitopes comprises less than twenty (20) epitopes in total.

In a particular embodiment, said plurality of epitopes comprises ten (10) to fifteen (15) epitopes in total.

One particular embodiment provides an isolated protein comprising thirteen (13) CMV CTL epitopes, such as shown in Table 2. In a preferred embodiment, at least one of the epitopes comprises the CMV amino acid sequence VTEHDTLLY (SEQ ID NO:11).

The full length, contiguous polyepitope protein comprises the amino acid sequence set forth in SEQ ID NOs:42-48 and shown in FIG. 15A-G.

It will also be appreciated that other CMV CTL epitopes may be used, such as described in International Publication WO 03/000720.

One particular embodiment provides an isolated protein comprising thirteen (13) EBV CTL epitopes, such as shown in Table 3. The full length, contiguous EBV polyepitope protein comprises the amino acid sequence set forth in SEQ ID NO:49 as shown in FIG. 15H.

It will also be further appreciated that other EBV CTL epitopes may be used such as described in International Publications WO 95/024925; WO 97/45444; WO 99/02550 and WO 04/041849.

The isolated polyepitope protein may further comprise one or a plurality of HLA

Class II restricted CTL epitopes.

It will be appreciated by a skilled person that epitope selected may be tailored to fit any population, race or other group of individuals.

Other criteria for inclusion within the herpesvirus polyepitope include those (i) having minimal or no sequence variants; (ii) selected from HLAs having minimal subtypes; (iii) having a high frequency of CTL responses in healthy seropositives; and (iv) based on epitope hydrophobic properties, wherein the novel sequential order of individual epitopes are arranged such that hydrophobicity is uniform distributed along the length of the polyepitope to assist inter cellular mobility.

Furthermore, it will be appreciated that the particular number and order of the constituent CTL epitopes may readily be altered while retaining broad HLA Class I-restricted immunogenicity.

In addition to the CTL epitopes, the isolated protein may further comprise intervening amino acids or amino acid sequences. Intervening amino acids or amino acid sequences may be present between at least two of the CTL epitope amino acid sequences, or between each adjacent CTL epitope amino acid sequence.

Suitably, the intervening amino acids or amino acid sequences are positioned or located relative to the CTL epitope amino acid sequences to enable proteasomal processing and for transporting the proteasome-generated, individual CTL epitope peptides into the endoplasmic reticulum (ER) for subsequent presentation with HLA-I molecules.

In one embodiment, the intervening amino acids or amino acid sequences are proteasome liberation amino acids or amino acid sequences.

Non-limiting examples of proteasome liberation amino acids or amino acid sequences are or comprise AD, K or R.

In an optional embodiment, the intervening amino acids or amino acid sequence are TAP recognition motifs. Typically, TAP recognition motifs may conform to the following formula: $(R/N:I/Q:W/Y)_n$ where n is any integer ≥ 1.

Non-limiting examples of TAP recognition motifs include RIW, RQW, NIW and NQY.

In a preferred form, CMV and/or EBV CTL epitopes are linked or joined by the proteasome liberation amino acid sequence and, optionally, the TAP recognition motif at the carboxyl terminus of each epitope.

Non-limiting examples of TAP recognition motifs, proteasome liberation amino

acids and their positioning relative to the CTL epitope amino acid sequences are shown in Table 1 and Table 2 and are also present in the polyepitope amino acid sequences shown in FIG. 1 (SEQ ID NO:58) and FIGS. 15A-H (SEQ ID NOS:42-49).

Surprisingly, once administered the exogenous protein comprising the intervening amino acids or amino acid sequences is processed by a novel, cellular TAP-independent, proteasome and autophagy dependent pathway. This results in HLA Class I-dependent presentation of the processed CMVepitopes to $CD8^+$ cytotoxic T cells.

Therefore, , the TAP amino acid sequences may be omitted or absent, in which case it is proposed or expected that the TAP-independent pathway could sufficiently process the isolated protein to enable presentation with HLA-I molecules.

In another embodiment, the isolated polyepitope protein may further comprise one or a plurality of $CD4^+$ helper T cell epitopes.

It will also be appreciated that the isolated protein described herein may be subjected to further modifications, variations and/or derivitizations without departing from the inventive concept.

Variations in amino acid sequence may be the result of naturally occurring sequence variation in a herpesvirus polyepitope protein.

It is well understood in the art that some amino acids may be changed to others with broadly similar properties without changing the nature of the activity of the isolated protein (conservative substitutions).

Typically, conservative substitutions are made so that amino acid properties such as charge, hydrophilicity, hydrophobicity and/or side chain size or "bulkiness" are retained or at least minimally altered.

Introduction of amino acid substitutions may be readily achieved during peptide synthesis or by mutagenesis of an encoding nucleic acid.

Non-limiting examples of nucleic acid mutagenesis methods are provided in Chapter 9 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel *et al.*, *supra*, Stemmer, 1994, Proc. Natl. Acad. Sci. USA **91** 10747, Shafikhani *et al.*, 1997, Biotechniques **23** 304, Jenkins *et al.*, 1995, EMBO J. **14** 4276-4287 and Zaccolo *et al.*, 1996, J. Mol. Biol. **255** 58 and kits such as QuickChange™ Site-Directed Mutagenesis Kit (Stratagene) and the Diversify™ random mutagenesis kit (Clontech).

Generally, the invention contemplates protein variants having at least 75%, preferably at least 80%, more preferably at least 85% or even more preferably at least 90, 91, 92, 93,

94, 95, 96, 97, 98 or 99% amino acid sequence identity with the constituent CTL epitope sequences, individually or in combination. In other embodiments, this may include conservative variations or substitutions of one (1), two (2) or three (3) amino acid residues of a CTL epitope.

The term "*sequence identity*" is used herein in its broadest sense to include the number of exact amino acid matches having regard to an appropriate alignment using a standard algorithm, having regard to the extent that sequences are identical over a window of comparison. Sequence identity may be determined using computer algorithms such as GAP, BESTFIT, FASTA and the BLAST family of programs as for example disclosed by Altschul *et al.*, 1997, Nucl. Acids Res. **25** 3389. A detailed discussion of sequence analysis can be found in Unit 19.3 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY Eds. Ausubel *et al.* (John Wiley & Sons Inc NY, 1995-1999).

As used herein, *"derivative"* proteins of the invention have been altered, for example by conjugation, fusion with additional protein sequences, by complexing with other chemical moieties or by post-translational modification techniques as would be understood in the art.

"*Additions*" of amino acids may include fusion with amino acid sequences of other proteins such as "*fusion partners*" or "*epitope tags*" which assist recombinant protein purification and/or identification.

Well known examples of fusion partners include, but are not limited to, glutathione-S-transferase (GST), Fc portion of human IgG, maltose binding protein (MBP) and hexahistidine (HIS_6), which are particularly useful for isolation of the fusion polypeptide by affinity chromatography. For the purposes of fusion protein purification by affinity chromatography, relevant matrices for affinity chromatography are glutathione-, amylose-, and nickel- or cobalt-conjugated resins respectively. Many such matrices are available in "kit" form, such as the QIAexpress™ system (Qiagen) useful with (HIS_6) fusion partners and the Pharmacia GST purification system.

Another fusion partner well known in the art is green fluorescent protein (GFP). This fusion partner serves as a fluorescent "tag" which allows the fusion protein of the invention to be identified by fluorescence microscopy or by flow cytometry. The GFP tag is useful when assessing subcellular localization of the fusion polypeptide of the invention, or for isolating cells which express the fusion polypeptide of the invention. Flow

cytometric methods such as fluorescence activated cell sorting (FACS) are particularly useful in this latter application.

Preferably, the fusion partners also have protease cleavage sites, such as for Factor X_a or Thrombin, which allow the relevant protease to partially digest the fusion protein of the invention and thereby liberate the recombinant protein of the invention therefrom. The liberated protein can then be isolated from the fusion partner by subsequent chromatographic separation.

Fusion partners according to the invention also include within their scope "*epitope tags*", which are usually short sequences for which a specific antibody is available. Well-known examples of epitope tags for which specific monoclonal antibodies are readily available include c-myc, influenza virus haemagglutinin and FLAG tags.

Other derivatives contemplated by the invention include, but are not limited to, modification to side chains, biotinylation, modification with fluorochromes, incorporation of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis, the use of crosslinkers and other methods which impose conformational constraints on the isolated protein of the invention. Examples of side chain modifications contemplated by the present invention include: modifications of amino groups such as by acylation; modification of carboxyl groups by carbodiimide activation via O-acylisourea formation followed by subsequent derivitization; sulfydryl group modification by methods such as performic acid oxidation to cysteic acid; formation of mercurial derivatives; formation of a mixed disulfides; alkylation of tryptophan residues; nitration of tyrosine residues; and modification of the imidazole ring of a histidine residue by alkylation; although without limitation thereto.

Examples of non-natural amino acids include but are not limited to, use of 4-amino butyric acid, 6-aminohexanoic acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 4-amino-3-hydroxy-6-methylheptanoic acid, t-butylglycine, norleucine, norvaline, phenylglycine, ornithine, sarcosine, 2-thienyl alanine and/or D-isomers of amino acids.

In another aspect, the invention provides an isolated nucleic acid encoding the aforementioned isolated protein of the invention.

The isolated nucleic acid of the invention may be useful for recombinant protein expression *in vivo* in an animal, or in a host cell for the purposes of subsequent recombinant protein purification.

It will be appreciated by persons skilled in the art that advantage may be taken of degeneracy in the genetic code to alter an encoding nucleotide sequence of an amino acid sequence.

In a particular example, a nucleotide sequence may be engineered according to codon preference or usage in an organism or cell type to thereby optimize encoded protein translation and expression in that organism or cell type.

The term *"nucleic acid"* as used herein designates single-or double-stranded mRNA, RNA, cRNA and DNA, said DNA inclusive of cDNA and genomic DNA.

Nucleic acids may comprise genetically-encoded bases such as adenine, guanine, cytosine, thymine and uracil, or modified bases such as inosine, methylinosine and methyladenosine, thiouridine and methylcytosine, although without limitation thereto.

The term *"recombinant"* as used herein means artificially produced through human manipulation of genetic material, such as involving techniques generally falling within the scope of *"recombinant DNA technology"* as is well understood in the art..

A *"polynucleotide"* is a nucleic acid having eighty (80) or more contiguous nucleotides, while an *"oligonucleotide"* has less than eighty (80) contiguous nucleotides.

A *"probe"* may be a single or double-stranded oligonucleotide or polynucleotide, suitably labelled for the purpose of detecting complementary sequences in Northern or Southern blotting, for example.

A *"primer"* is usually a single-stranded oligonucleotide, preferably having 15-50 contiguous nucleotides, which is capable of annealing to a complementary nucleic acid "template" and being extended in a template-dependent fashion by the action of a DNA polymerase such as Taq polymerase, RNA-dependent DNA polymerase or SequenaseTM.

An *"amplification product"* refers to a nucleic acid product generated by nucleic acid amplification techniques.

An embodiment of an isolated nucleic acid comprises a nucleotide sequence set forth in any one of SEQ ID NOS: 50-57 and as shown in FIG. 15.

Also contemplated according to the present invention are isolated nucleic acids that encode variants and/or derivatives of the isolated protein as hereinbefore described.

In some embodiments, nucleic acid variants encode isolated protein variants as hereinbefore described.

In other embodiments, nucleic acid variants encode isolated proteins disclosed herein, or variants thereof, said nucleic acid variants adopting nucleotide sequence changes

due to redundancy in the genetic code. In one particular form, such variants are "codon optimized" for expression in a particular organism or cell type.

Isolated nucleic acid variants may hybridize with an isolated nucleic acid encoding an isolated polyepitope protein under high stringency wash conditions.

High stringency conditions include and encompass:-

(i) from at least about 31% v/v to at least about 50% v/v formamide and from at least about 0.01 M to at least about 0.15 M salt for hybridisation at 42°C, and at least about 0.01 M to at least about 0.15 M salt for washing at 42°C;

(ii) 1% BSA, 1 mM EDTA, 0.5 M $NaHPO_4$ (pH 7.2), 7% SDS for hybridization at 65°C, and (a) 0.1 x SSC, 0.1% SDS; or (b) 0.5% BSA, 1mM EDTA, 40 mM $NaHPO_4$ (pH 7.2), 1% SDS for washing at a temperature in excess of 65°C for about one hour; and

(iii) 0.2 x SSC, 0.1% SDS for washing at or above 68°C for about 20 minutes.

In another embodiment, isolated nucleic acid variants may have at least 60%, 70%, 75%, 80%, 85%, 90%, or 95% sequence identity with a reference nucleic acid. Non-limiting examples of reference nucleic acids comprise a nucleotide sequence set forth in any one of SEQ ID NO:50-57.

Another aspect of the invention provides a genetic construct comprising an isolated nucleic acid of the invention, or a variant thereof.

The genetic construct may facilitate propagation, cloning and/or expression of the isolated nucleic acid.

In a preferred form, the genetic construct is an expression construct comprising an isolated nucleic acid of the invention operably linked to one or more regulatory sequences present in an expression vector.

An "*expression vector*" may be either a self-replicating extra-chromosomal vector such as a plasmid, or a vector that integrates into a host genome. Suitably, the expression vector provides said one or more regulatory nucleotide sequences. By *"operably linked"* is meant that said regulatory nucleotide sequence(s) is/are positioned relative to the recombinant nucleic acid of the invention to initiate, regulate or otherwise control transcription.

Regulatory nucleotide sequences will generally be appropriate for the host cell used for expression. Numerous types of appropriate expression vectors and suitable regulatory sequences are known in the art for a variety of host cells.

Typically, said one or more regulatory nucleotide sequences may include, but are not limited to, promoter sequences, leader or signal sequences, ribosomal binding sites, transcriptional start and termination sequences, translational start and termination sequences, and silencer, enhancer or activator sequences.

With regard to promoters, constitutive promoters (such as CMV, SV40, vaccinia, HTLV1 and human elongation factor promoters) and inducible/repressible promoters (such as *tet*-repressible promoters and IPTG-, metallothionin- or ecdysone-inducible promoters) are well known in the art and are contemplated by the invention. It will also be appreciated that promoters may be hybrid promoters that combine elements of more than one promoter, such as but not limited to the SRα promoter which is a hybrid between elements of HTLV1 and SV40 promoters.

Preferably, said expression construct also includes one or more selectable markers suitable for the purposes of selection of transformed bacteria (such as *bla*, *kanR* and *tetR*) or transformed mammalian cells (such as hygromycin, G418 and puromycin).

Expression constructs may be transfected, transformed or otherwise introduced into host cells by any of a number of well known techniques including, but not limited to, transformation by heat shock, electroporation, DEAE-Dextran transfection, microinjection, liposome-mediated transfection, calcium phosphate precipitation, protoplast fusion, microparticle bombardment, viral transformation and the like.

The conditions appropriate for protein expression will vary with the choice of expression vector and the host cell. This is easily ascertained by one skilled in the art through routine experimentation.

Suitable host cells for expression may be prokaryotic or eukaryotic, such as bacterial cells inclusive of *Escherichia coli (DH5α* for example*)*, yeast cells such as *Pichia pastoris*, Sf9 cells utilized with a baculovirus expression system, mammalian cell lines such as human embryonic kidney (HEK) 293 cells, CHO cells, COS cells, CV1 cells, Jurkat and NIH3T3 cells although without limitation thereto.

Another aspect of the invention provides a method of producing the isolated protein disclosed herein in recombinant form, said method including the steps of expressing the isolated protein in a host cell as hereinbefore described and at least partly purifying the

isolated protein under conditions that maintain the isolated protein in a substantially non-aggregated form.

By "*non-aggregated*" in this context is meant that a substantial portion of the isolated protein is in a soluble form in aqueous solution, typically in the absence of denaturing agents such as urea, SDS or guanidinium chloride.

Because of the hydrophobic nature of CTL epitopes and TAP sequences, expression of the isolated protein in bacteria tends to result in aggregated protein in the form of inclusion bodies (IBs). While IBs may be solubilised and the recombinant protein purified using an affinity matrix (such as a Ni-NTA matrix), isolated proteins comprising twenty (20) CMV CTL epitopes were resistant to this treatment. Accordingly, a preferred form of the invention provides an isolated protein comprising less than twenty (20) CMV and/or EBV CTL epitopes. Given that each CMV CTL epitope in Tables 1 and 2 comprises 8-13 amino acids, less than twenty (20) CMV CTL epitopes is equivalent to less than 160-240 constituent, epitope amino acids.

Furthermore, maintaining the purified recombinant protein in a soluble form is difficult and has been a contributing factor to the inability to successfully administer polyepitope proteins as an exogenous protein that elicits a CD8+ CTL response. As described in more detail in the Examples, a compatible buffer system to maintain indicated that the isolated polyepitope proteins require MES or a glycine buffer at an acidic pH to remain soluble.

Accordingly, one embodiment of the invention provides a method of producing the isolated protein disclosed herein in recombinant form, said isolated protein having fewer than twenty (20) CMVCTL epitopes or 160-240 constituent epitope amino acids, said method including the steps of expressing the isolated protein in a bacterial host cell as hereinbefore described and at least partly purifying the isolated protein under conditions that maintain the isolated protein in a substantially non-aggregated form, wherein the conditions include maintaining the isolated recombinant protein in an MES buffer or a glycine buffer under acidic conditions.

Acidic conditions may be any pH below 7, preferably in the range pH 2-6 or more preferably in the range of about pH 2.5 to about pH 5.6.

General guidance on producing recombinant proteins may be found in standard protocols as for example described in Sambrook *et al.*, MOLECULAR CLONING. A Laboratory Manual (Cold Spring Harbor Press, 1989), in particular Sections 16 and 17;

CURRENT PROTOCOLS IN MOLECULAR BIOLOGY Eds. Ausubel *et al.*, (John Wiley & Sons, Inc. NY USA 1995-2001), in particular Chapters 10 and 16; and CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et al.*, (John Wiley & Sons, Inc. NY USA 1995-2001, in particular Chapters 1, 5 and 6.

In embodiments relating to expression constructs for administration to humans, the expression construct of the invention is suitable for use as a DNA vaccine.

In particular forms, the expression construct of the invention may be a construct that utilizes an expression and delivery vector of viral origin, such as pox viruses and adenoviruses or a DNA plasmid vector.

When used as a vaccine delivery system, expression constructs of viral origin may be administered to an animal in the form of VLPs or as a "naked" nucleic acid construct.

In one particular embodiment, the expression construct according to this embodiment comprises a vaccinia virus promoter, such as the p7.5 promoter present in a plasmid vector. For example, production of a TK- recombinant vaccinia virus using marker rescue recombination as provided in Khanna *et al.*, 1992. J Exp Med. **176** 169.

In a more preferred embodiment, the invention provides an adenovirus-based expression construct for use in a vaccine delivery system. Adenovirus-based constructs are capable of infecting a broad spectrum of mammalian and human cells, including both quiescent and proliferating cell types.

Such adenovirus-based expression constructs may comprise a constitutive or inducible/repressible promoter such as by way of a tetracycline inducible/repressible system.

One form of the adenovirus-based expression construct is derived from a replication-incompetent A5 adenovirus lacking at least an E1 gene.

A particular form is the Ad5/F35 adenovirus-based expression construct and vaccine delivery system is provided in detail hereinafter. Reference is also made to Yotdna *et al.*, 2001, Gene Therapy **8** 930, in relation to the Ad5/F35 embodiment of adenovirus expression vectors.

It will be appreciated that the isolated protein of the invention, isolated nucleic acids and expression constructs encoding same may be useful in therapeutic and/or prophylactic treatment of a herpesevirus-associated disease or condition such as a Cytomegalovirus-associated or Epstein-Barr-associated disease and/or condition in animals, preferably humans.

In humans, CMV infection can cause a mononucleosis-like syndrome with prolonged fever, and/or a mild hepatitis. In certain high-risk groups, disease can be more severe, such as during infection of the unborn baby during pregnancy, in people who work with children, and in immunocompromised persons, such as the aged, organ transplant recipients and persons infected with human immunodeficiency virus (HIV). CMV may also be associated with some cancers such as glioma. The invention therefore provides pharmaceutical compositions and/or methods of prophylactic or therapeutic treatment of CMV infection, preferably in humans.

EBV infection can cause serious mononucleosis and is also associated with a variety of cancers and possibly autoimmune disorders. The invention therefore provides pharmaceutical compositions and/or methods of prophylactic or therapeutic treatment of CMV infection, preferably in humans.

Such pharmaceutical compositions and methods are suitable for delivery of the isolated protein in recombinant form, or encoded by an expression construct such as in a viral delivery vector. In this regard, it will be appreciated that the pharmaceutical composition may comprise separate isolated proteins respectively comprising CMV and EBV CTL epitopes or may comprise a single isolated protein comprising both EBV and CMV epitopes.

Suitably, pharmaceutical compositions further comprise a pharmaceutically-acceptable carrier, diluent or excipient.

By "*pharmaceutically-acceptable carrier, diluent or excipient*" is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in systemic administration. Depending upon the particular route of administration, a variety of carriers, well known in the art may be used. These carriers may be selected from a group including sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulfate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline and salts such as mineral acid salts including hydrochlorides, bromides and sulfates, organic acids such as acetates, propionates and malonates and pyrogen-free water.

A useful reference describing pharmaceutically acceptable carriers, diluents and excipients is Remington's Pharmaceutical Sciences (Mack Publishing Co. N.J. USA, 1991) which is incorporated herein by reference.

Any safe route of administration may be employed for providing a patient with the composition of the invention. For example, oral, rectal, parenteral, sublingual, buccal,

intravenous, intra-articular, intra-muscular, intra-dermal, subcutaneous, inhalational, intraocular, intraperitoneal, intracerebroventricular and transdermal administration may be employed.

Dosage forms include tablets, dispersions, suspensions, injections, solutions, syrups, troches, capsules, suppositories, aerosols, transdermal patches and the like. These dosage forms may also include injecting or implanting controlled releasing devices designed specifically for this purpose or other forms of implants modified to act additionally in this fashion. Controlled release of the therapeutic agent may be effected by coating the same, for example, with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, the controlled release may be effected by using other polymer matrices, liposomes and/or microspheres.

Preferred pharmaceutical compositions are "*immunogenic compositions*" that elicit a CT: response to thereby provide prophylactic and/or therapeutic treatment of herpesvirus (e.g CMV and/or EBV) responsive to such immunotherapy, without necessarily eliciting a protective immune response.

In a preferred form, the immunogenic composition may be a vaccine for eliciting a protective $CD8^+$ CTL-based immune response in a human subject that protects against CMV infection, or treats an existing herpesvirus (*e.g* CMV and/or EBV) infection.

In one particular embodiment, the pharmaceutical composition, inclusive of immunogenic compositions and vaccines, comprises the isolated protein disclosed herein and said pharmaceutically-acceptable carrier, diluent or excipient.

As will be described in more detail in the Examples, the isolated protein comprising a plurality of CMV and/or EBV CTL epitopes are highly efficient in generating CMV-specific $CD8^+$ T cell responses in virus healthy carriers. Furthermore, expanded $CD8^+$ T cells demonstrated strong expression of IFN-γ, TNF, MIP-1β and CD107a following stimulation with the protein. It is proposed that these functional characteristics of the $CD8^+$ T cells are important for predicting the efficacy of CTL- mediated immune responses and virus clearance.

Alternative embodiments provide a pharmaceutical composition, inclusive of immunogenic compositions and vaccines, comprising a nucleic acid expression construct, inclusive of DNA vaccines, encoding the isolated protein disclosed herein and said pharmaceutically-acceptable carrier, diluent or excipient. According to this alternative

embodiment, the pharmaceutical composition, inclusive of immunogenic compositions and vaccines, may comprise an expression construct that utilizes a viral vector such as an adenoviral vector or pox virus-derived vector as hereinbefore described.

Any suitable procedure is contemplated for producing such vaccines. Exemplary procedures include, *eg.*, those described in New Generation Vaccines (1997, Levine *et al.*, Marcel Dekker, Inc. New York, Basel, Hong Kong) which is incorporated herein by reference.

Pharmaceutical compositions, immunogenic compositions, vaccines and/or methods of prophylactic or therapeutic treatment may include one or more immunostimulatory molecules or adjuvants for administration to the animal.

Suitable immunostimulatory molecules and adjuvants include, but are not limited to: TLR agonists, lipopolysaccharide and derivatives thereof such as MPL, Freund's complete or incomplete adjuvant, hexadecylamine, octadecylamine, octadecyl amino acid esters, lysolecithin, dimethyldioctadecylammonium bromide, N,N-dicoctadecyl-N′, N′bis(2-hydroxyethyl-propanediamine), methoxyhexadecylglycerol, and pluronic polyols; polyamines such as pyran, dextransulfate, poly IC carbopol; peptides such as muramyl dipeptide and derivatives, dimethylglycine, tuftsin; oil emulsions; and mineral gels such as aluminum phosphate, aluminum hydroxide or alum; lymphokines, Imiquimod, Guardiquimod, QuilA and immune stimulating complexes (ISCOMS).

Pharmaceutical compositions, immunogenic compositions, vaccines and/or methods of prophylactic or therapeutic treatment may include one or more other TLR agonists for administration to the animal. Preferably, the one or more TLR agonists include a TLR4 agonist and/or a TLR9 agonist.

Preferred TLR4 agonists are lipolopysacchardides (LPS) or derivatives or components of LPS. These include Monophosphoryl lipid A (MPL®) derived from *Salmonella minnesota* and synthetic TLR4 agonists such as aminoalkyl glucosaminide phosphates (AGPs). A preferred TLR4 agonist is MPL.

TLR9 recognizes specific unmethylated CpG oligonucleotides (ODN) sequences that distinguish microbial DNA from mammalian DNA. CpG ODNs oligonucleotides contain unmethylated CpG dinucleotides in particular sequence contexts (CpG motifs). These CpG motifs are present at a 20-fold greater frequency in bacterial DNA compared to mammalian DNA. Three types of stimulatory ODNs have been described: type A, B and C.

Non-limiting examples of TLR9 agonists include CpG ODN1826, CpG ODN2006, CpG ODN2216 and CpG ODN2336, although without limitation thereto.

Generally, pharmaceutical compositions, immunogenic compositions, vaccines and/or methods of prophylactic or therapeutic treatment may employ any safe route of administration may be employed for providing a patient with the composition of the invention. For example, oral, rectal, parenteral, sublingual, buccal, intravenous, intra-articular, intra-muscular, intra-dermal, subcutaneous, inhalational, intraocular, intraperitoneal, intracerebroventricular, transdermal and the like may be employed. Intra-muscular and subcutaneous injection is appropriate, for example, for administration of immunogenic compositions, proteinacious vaccines and DNA vaccines.

With regard to methods of treatment of a herspesvirus infection such as a CMV or EBV infection and/or a disease or condition associated with, or resultant from a CMV or EBV infection, the invention contemplates adoptive immunotherapy.

Preferably, although not exclusively, the invention contemplates adoptive immunotherapy using autologous CTLs produced *in vitro*.

Current methods for expanding herpesvirus (*e.g* CMV or EBV) CTLs are very difficult and are often based on either using a CMV lysate or individual peptide epitopes.

The isolated protein of the invention is expected to be more advantageous than either of these prior art approaches by facilitating expansion of broadly focussed T cell responses.

Accordingly, a method of expanding herpesvirus-specific CTLs for adoptive immunotherapy, includes the steps of:

(a) contacting one or more cells isolated from an animal with the isolated protein disclosed herein; and

(b) culturing said one or more cells to thereby expand herpesvirus-specific CTLs from said one or more cells.

Furthermore, a method of adoptive immunotherapy includes the step of administering said herpesvirus-specific CTLs produced at step (b) to an animal to thereby prophylactically or therapeutically treat a herpesvirus infection of said animal.

Preferably, the animal is a mammal, such as a human.

In one embodiment, the invention provides a method of autologous adoptive immunotherapy in a human including the steps of:

(A) contacting one or more cells isolated from a human with an isolated protein disclosed herein;

(B) culturing said one or more cells to thereby expand herpesvirus -specific CTLs from said one or more cells; and

(C) administering said herpesvirus-specific CTLs to said human to thereby prophylactically or therapeutically treat a herpesvirus infection of said animal.

In particular embodiments the herpesvirus is CMV or EBV.

In order that the invention may be readily understood and put into practical effect, particular embodiments will now be described by way of the following non-limiting examples.

EXAMPLES

EXAMPLE 1

Purification and immunogenicity of CMV polyepitope protein

Materials and Methods

Construction of CMV polyepitope vectors

A series of CMV polyepitope inserts were designed to encode multiple HLA class I restricted T-cell epitopes from five different antigens (pp65, IE-1, pp50, pp150 and gB). These polyepitope sequences encoded 13, 14, 15 or 20 different HLA class I-restricted $CD8^+$ epitopes (see Table 1).

The polyepitope sequences were designed in such a way that each epitope sequence was preceded by a proteasome liberation amino acid sequence (AD or K or R) and a TAP recognition motif (RIW, RQW, NIW or NQY). In addition, a hexa-histidine tag was inserted at the c-terminus of each polyepitope protein to allow purification using a nickel-nitrilotriacetic acid (Ni-NTA) column. The amino acid sequence of each construct was translated into DNA sequence based on *E. coli* codon utilisation and inserts were synthetically constructed (DNA2.0, California, USA) and cloned into an expression plasmid (pJexpress 404) under an isopropyl –β-D-thiogalactopyraniside (IPTG) inducible promoter. These synthetically designed polyepitope constructs were transformed into chemically competent *E. coli* DH5α (Invitrogen, Carlsbad, CA, USA) and plasmids were purified using a QIAGEN maxi prep kit (QIAGEN, Hilden, Germany).

Protein expression

Chemically competent *E. coli* BL21 (DE3) pLysS (Invitrogen, California, USA) was transformed with the CMV polyepitope expression vector. Transformed cells were plated on Luria Bertani (LB) agar supplemented with 100 µg/mL of ampicillin (LB-Amp) and plates were incubated overnight at 37°C. An isolated colony was picked and inoculated into 10 ml of LB-Amp broth and grown in a shaker at 37°C and 200 rpm overnight. A small amount of overnight culture was inoculated into 50 mL of LB-Amp broth and grown for 12 hours, then 1% of culture was transferred into 2 L of LB-Amp broth that was then was grown until the O.D. reached 0.6 at 600nm. CMV polyepitope protein induction was carried out by adding 1 mM/mL of IPTG. These cells were allowed to grow for an additional 4 hours and protein expression levels were determined by analysing un-induced and induced samples on 12-15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

CMV polyepitope protein purification

At the end of the induction phase, *E. coli* cultures were harvested by centrifugation at 10,000 rpm for 15 minutes, the cell pellet was resuspended in 80 mL of lysis buffer (25 mM Tris pH 7.4, 0.5% TritonX100, 150 mM NaCl, 0.5 mg/mL lysozyme) supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany) and incubated on the ice for 30 minutes. Cell lysis was carried out by sonication on ice for 4 x 5 minutes cycles with a 10 minute break between each cycle. The lysate was centrifuged at 13,000 rpm for 30 minutes and supernatant and pellet fractions were analysed using SDS-PAGE. Since the majority of the protein was found in the pellet fractions in the form of inclusion bodies (IBs), IBs were washed once with lysis buffer (without lysozyme) under stirring for two hours at RT and solubilised in 150 mL of solubilisation buffer (100 mM NaH_2PO_4, 10 mM Tris, 8 M urea pH 8.0) overnight at 4°C. The soluble protein was clarified by centrifugation at 13,000 rpm for 30 minutes and supernatant was used for purification of polyepitope proteins.

To purify the CMV polyepitope proteins we used 5 mL of Ni-NTA (QIAGEN, Hilden, Germany) metal-affinity chromatography matrix. The matrix was washed with 5 column volumes of distilled water followed by equilibration with 3 column volumes of solubilisation buffer. The soluble protein was loaded on the column and the flow rate was

adjusted to 1 mL/minute. The unbound protein and impurities were washed-out with 10 column volumes of wash buffer 1 (100 mM NaH_2PO_4, 10 mM Tris, 8 M urea pH 6.3) and 20 column volumes of wash buffer 2 (100 mM NaH_2PO_4, 10 mM Tris, 8 M urea pH 5.9). The bound protein was eluted with elution buffer (100 mM NaH_2PO_4, 10 mM Tris, 8 M urea pH 4.3) and the eluted fractions were analysed using SDS-PAGE. The positive fractions were pooled together and CMV polyepitope protein estimation was carried out using a Bradford assay kit (Bio-Rad, Hercules, California, USA) following the manufacturer's instructions. Purified protein was subjected to a solubility test (to identify the right buffer composition for storing the protein in the soluble form) in which 80 µL of purified protein was diluted into 800 µL of various compositions of buffers with different pH ranges. These include (a) 25 mM MES buffer pH 5.6; (b) 25 mM MES buffer pH 3.2; (c) 25 mM MES pH 4.5; (d) 25 mM MES (2-(*N*-morpholino) ethanesulfonic acid) pH 4.5 and 400 mM L-arginine; (e) 10 mM Tris and 100 mM NaH_2Po_4 pH 4.3; (6) 10 mM Tris, 100 mM NaH_2Po_4 and 400 mM L- arginine pH 4.3; (f) PBS, 50 mM L-arginine and 50 mM L- glutamic acid pH7.4; (g) diluted in water; (h) 100 mM glycine buffer pH 2. These samples were incubated at 4°C overnight; spun at 13,000 rpm for 25 minutes and supernatant fractions were analysed using SDS-PAGE. CMV polyepitope protein was dialysed against 25 mM MES buffer at pH 5.6. The CMV polyepitope protein was concentrated using Ultracel-10K spin columns (Millipore, County Cork, Ireland) followed by sterile filtration using 0.22µ membrane filter, total protein was estimated using BIO-RAD Bradford protein assay kit and various concentrations of CMV polyepitope protein was analysed using SDS-PAGE to determine the final purity of polyepitope protein. The purified protein was stored in 1 ml aliquots at -70°C.

In vitro stimulation and expansion of CMV specific T-cells from healthy donors using polyepitope proteins

Peripheral blood mononuclear cells (PBMC) from healthy virus carriers were incubated with 25 µg of purified polyepitope protein at 37°C, 6.5% CO_2 for 2 hours. After incubation, these PBMC were mixed with un-pulsed PBMC and resuspended in RPMI 1640 medium supplemented with 10% FCS (referred to as growth medium). These cells were cultured in a 24 well plate for 10 days at 37°C, 6.5% CO_2. On days 3 and 6, cultures were supplemented with 1 mL of growth medium containing 100 U of recombinant IL-2. The T cell specificity of these *in vitro* expanded cells was assessed using a standard ICS

assay. In addition, T cells in these cultures were also assessed for polyfunctional capacity using multi-parameter flow cytometry.

Analysis of processing and presentation of $CD8^+$ T cell epitopes from CMV polyepitope protein by human cells

Epstein-Barr virus (EBV) transformed LCLs and HEK 293 cells were used as antigen presenting cells in these assays. These cells were pulsed with 25-100 µg of CMV polyepitope protein for two hours at 37°C, 6.5% CO_2 and then washed twice with RPMI 1640 medium, resuspended in growth medium and incubated overnight at 37°C, 6.5% CO_2. After overnight incubation, antigen presenting cells were exposed to CMV-specific T cells at a responder to stimulator ratio of 4:1 for four hours at 37°C, 6.5% CO_2 and T cells assessed for cytokine expression using ICS assays.

Enzyme inhibition assays

To assess the role of various proteases involved in the processing of CMV polyepitope protein, LCLs were pre-treated with different inhibitors and then used as antigen presenting cells. These inhibitors were specifically targeted to inhibit lysosomes/endosome acidification(80 µM chloroquine and 10 mM Bafilomycin A1), the recycling pathway (200 µM primaquine), cysteine proteases (100 µM leupeptin and 100 µM E64), acid proteases (pepstatin A), autophagy mediators (10 mM 3-methyladenine(3-MA)), the proteasome complex (10 µM lactacystine, 1 µM epoxomicin and MG132), golgi transport (1 µg/mL brefeldin A and 0.7 µg/mL monensin) or aminopeptidase enzymes (30 µM leucinethiol with 0.5mM dithiothreitol (DTT)). Following pre-treatment with these inhibitors, cells were incubated with 25 µg of CMV polyepitope protein for two hours at 37°C, 6.5% CO_2, washed twice with RPMI 1640 medium, resuspended in growth medium and incubated overnight at 37°C, 6.5% CO_2. After overnight incubation, cells were exposed to CMV-specific T cells at a responder to stimulator ratio of 4:1 for four hours at 37°C, 6.5% CO_2 and T cells assessed for cytokine expression using ICS assays.

Silencing of Atg12 or Sec61 with short hairpin RNA (shRNA)

Lentivirus based vectors encoding ATG12 shRNA (clone ID NM_004707.2-485s1c1,

(CCGGTGTTGCAGCTTCCTACTTCAACTCGAGTTGAAGTAGGAAGCTGCAACAT TTTT; SEQ ID NO:59) or Sec61 shRNA (clone ID NM_006808.2-410s1c1, CCGGCCCAACATTTCTTGGACCAAACTCGAGTTTGGTCCAAGAAATGTTGGGTT TTTTG; SEQ ID NO:60) were obtained from Sigma-Aldrich in an *E.coli* host. Plasmid encoding shRNA was purified using the large scale plasmid purification kit (Qiagen, Hilden, Germany). Lentivirus was produced in HEK293T cells by cotransfecting the shRNA vector or control vector (pLKO.1puro) with a packaging vector, pHR8.2ΔR, and an envelope vector, pCMV-VSV-G (vesicular stomatitis virus glycoprotein G). Following 48 and 72 hours of transfection, Lentivirus containing supernatant was harvested, 0.45μm filtered, and stored at -80°C. Transduction was performed by resuspending $3x10^5$ CEM.T1, CEM.T2 cells or LCLs in 1mL of lentivirus containing supernatant and centrifuging for 30 minutes at 800g and32°C. Puromycin (1μg/mL) was added 48 hours after transduction. To generate complete knock down cells were reinfected with the identical lentivirus vector on day 10 and cells were used for downstream assays after 5-7 days of transduction.

Western blotting

Western blot analysis was performed as previously described (Ausubel 1995). Briefly, lentivirus shRNA infected cells were washed in PBS and lysed with RIPA buffer (Thermo Scientific, Rockford, IL, USA) on ice according to the manufacturer's instructions. Protein was quantified using a DC protein assay kit (Bio-Rad laboratories, Hercules, CA, USA). Lysate was mixed with SDS-PAGE loading buffer and resolved on 12-15% SDS-PAGE gels, then transferred to a nitrocellulose membrane (using a Mini Trans-Blot apparatus (Bio-Rad, CA, USA) in pre-chilled transfer buffer (1X Tris-glycine buffer containing 20% methanol) at 100V for 1 hour. Following transfer the nitrocellulose membrane was washed three times in wash buffer (PBS containing 0.05% V/V Tween-20), then incubated in blocking buffer (PBS containing 5% skim milk) for 1 hour at room temperature on a shaker. The membrane was incubated in rabbit anti-Sec61 (Thermo Scientific, Australia) or rabbit anti-ATG12 (Cell Signaling Technology, Danvers, MA) primary antibody solution (diluted in blocking buffer) overnight at 4°C on a shaker. The membrane was washed 6 times with wash buffer for 10 minutes each wash, then incubated with sheep anti-rabbit conjugated to horseradish peroxidase (Chemicon, Australia) secondary antibody (diluted in blocking buffer) for 1 hour at room temperature. The

nitrocellulose membrane was washed in wash buffer, incubated with ECL reagent (Merck, Darmstadt, Germany) and protein visualised on an X-ray film.

Statistical analysis

Statistical analyses were carried out using Graph Pad software or Microsoft Office Excel 2007. For $CD8^+$ T cell responses, the means ± SD were calculated and *p* values were determined using the Student's t-test. Error bars represent S.E.M. Where indicated with *, ** and *** represents statistically significant with $p<0.05$, $p<0.01$ and $p<0.001$ respectively when compared to the controls.

Results

Purification and characterisation of CMV polyepitope protein

CMV polyepitope inserts encoding 13, 14, 15 or 20 minimal $CD8^+$ T cell epitopes were designed as outlined in Figure 1. A comprehensive list of CMV epitopes included in each of these polyepitope sequences are presented in Table 1. These CMV polyepitope constructs were transformed into *E. coli*, protein expression conditions were optimised and analysed on SDS-PAGE. Results obtained from these experiments showed that CMV polyepitope protein (13, 14, 15 and 20mer) can be successfully expressed using a bacterial expression system under an IPTG inducible promoter at 37° C (Fig. 2A & B). Because of the hydrophobic nature of the linear $CD8^+$ T cell epitopes, the CMV polyepitope protein was aggregated in the form of inclusion bodies (IBs, data not shown). These IBs were solubilised and CMV polyepitope proteins from constructs encoding 13, 14 or 15 epitopes were purified using Ni-NTA matrix. This one step purification process allowed us to purify these CMV polyepitope proteins to homogeneity (Fig. 3A-C). However, purification of CMV polyepitope 20mer was not successful, despite using two different denaturing agents, 8M urea and 6M guanadine hydrochloride, to solubilise the IBs. Following solubilisation CMV polyepitope the 20mer protein remained in the pellet fraction; and no protein was detected in the elution fractions (Fig. 3D). The data obtained from the solubility test to identify a compatible buffer system to maintain indicated that CMV polyepitope proteins require MES or glycine buffers at an acidic pH to remain soluble (Fig. 4A). Following CMV polyepitope purification, various concentrations of protein were analysed on SDS-PAGE to check integrity. Data presented in Figure 4B-D, shows minimal impurities and the

molecular weights of the recombinant polyepitope proteins were approximately 19, 21 and 25 kDa which matched with the theoretically calculated molecular weight of the 13, 14 and 15mer polyepitope respectively. This one step purification step allowed us to obtain 80 mg of the 13mer, 4 mg of the 14mer and 15 mg of the 15mer protein from 2 L of culture.

Ex vivo *expansion of CMV epitope specific CD8$^+$ T cells from PBMC following stimulation with polyepitope protein*

To evaluate the immunogenicity of the CMV polyepitope proteins, we performed several *in vitro* experiments using various HLA typed CMV-seropositive donor PBMC to expand CMV specific CD8$^+$ T cells. PBMC from healthy donors were stimulated *ex vivo* with purified CMV polyepitope proteins and then assessed for antigen specificity by ICS assay and compared with *ex vivo* responses. The data obtained from these experiments showed that 13, 14 and 15mer CMV polyepitope proteins induced a rapid expansion of CMV specific CD8$^+$ T cell specific for the epitopes included in the polyepitope (Fig. 5A). In most cases dominant CD8$^+$ T cell responses were against multiple epitopes included in the CMV polyepitope. For example, data presented in Figure 5A shows that a considerable increase in the percentage of CMV specific CD8$^+$ T cells against the multiple epitopes from individual donor PBMCs. In addition, we also showed that all epitopes within the CMV polyepitope were capable of expanding CMV specific CD8$^+$ T cells from PBMC and these responses ranged from 2 to 40% of the total CD8$^+$ T cells (Fig. 5B & 5C). Of particular interest was the QIK epitope as our *in vitro* expansion studies showed that T cells specific for this epitope can be expanded following stimulation with the polyepitope protein (Fig. 5B). In contrast, minimal expansion of QEF-specific T cells was observed suggesting that this epitope may not be efficiently processed by human cells. These results clearly demonstrate that CD8$^+$ T cell epitopes included in the polyepitope proteins can be efficiently processed and presented by human cells and sensitization of human PBMC with polyepitope protein induces the rapid expansion of CMV-specific T cells.

CD8$^+$ T cells expanded following stimulation with polyepitope proteins display polyfunctional profile

A large body of documented evidence suggest that polyfunctional CD4$^+$ and CD8$^+$ T cell responses are crucial in providing protection against a range of viral and microbial pathogens (Betts, Gray et al. 2006; Darrah, Patel et al. 2007; Millington, Innes et al. 2007).

In addition, in the context of CMV, polyfunctional $CD8^+$ T cells protect against high levels of viral replication after liver transplant (Nebbia, Mattes et al. 2008). These observations clearly highlight that polyfunctional $CD8^+$ T cell responses are a prerequisite for the development of a potent CMV vaccine. In our subsequent experiments, we analysed effector functions of CMV specific $CD8^+$ T cells expanded by polyepitope proteins. These analyses were designed to assess the ability of these effector cells to perform cytolytic function (CD107a mobilization) and express multiple cytokines (IFN-γ, TNF and MIP-1β). Representative data from one of these analyses is presented in Figure 6. The majority of the CMV-specific $CD8^+$ T cells expanded with the polyepitope displayed strong cytolytic function (as indicated by CD107a mobilization) and expressed multiple cytokines ($IFN\gamma^+$, TNF^+ and $MIP1\beta^+$).

Rational design of the CMV polyepitope constructs with and without linkers, protein expression, and purification

To delineate the precise role of spacer sequences in the processing and presentation of polyepitope proteins we have designed to encode 13 minimal $CD8^+$ T cell epitopes without (CMVpoly) and with proteasome linkers (CMVpoly-PL) (Figure 7A & 7B). The CMV polyepitope constructs were transformed into *E. coli,* protein expression conditions were optimised, and polyepitope proteins were purified using Ni-NTA chromatography. Results obtained from these experiments showed that both the CMVpoly and CMVpoly-PL could be successfully expressed and purified to homogeneity using a bacterial expression system.

In vitro evaluation of immunogenicity of CMV polyepitope proteins with and without linkers

To investigate the processing and presentation of the CMVpoly, CMVpoly-PL and CMVpoly-PTL proteins, we incubated human lymphoblastoid cell lines (LCLs) overnight with CMVpoly, CMVpoly-PL and CMVpoly-PTL, and then assessed the activation of a panel of CMV-specific T cells using intracellular IFN-γ analysis. Representative FACS plots presented in Figure 8A shows that HLA A2-restricted NLV (pp65), HLA A1-restricted VTE (pp50), HLA B7-restricted RPH and TPR (pp65) epitopes from CMVpoly-PL or PTL were more efficiently processed and presented to CMV-specific T cells compared to LCLs pulsed with CMVpoly. More importantly, the activation of CMV-

specific T cells were significantly higher following stimulation with LCL pulsed with CMVpoly-PL or CMVpoly-PTL compared to CMVpoly (Figure 8B). Collectively, these data indicate that to enhance the processing and presentation of the exogenously delivered polyepitope proteins to the antigen-specific $CD8^+$ T cells requires proteasome and/or TAP linkers between the epitopes

$CD8^+$ T cell epitopes from the polyepitope protein are cross-presented through a TAP-independent pathway but involves proteasome and the autophagy dependent pathway

To delineate the precise pathway for the processing and presentation of $CD8^+$ T cell epitopes from the exogenously loaded polyepitope protein in the next set of experiments we pulsed polyepitope protein in TAP^+ (CEM.T1) and TAP^- (CEM.T2 and CEM.T2-HLA B7) LCLs and then exposed these cells to CMV-specific T cells. Data presented in Figure 9 shows that both TAP^+ and TAP^- B cells can efficiently present $CD8^+$ T cell epitopes from the polyepitope protein. To delineate the mechanisms of polyepitope presentation we used CEM.T1 and CEM.T2 cells as antigen presenting cells to stimulate HLA A2 restricted NLV-specific $CD8^+$ T cells. These antigen presenting cells were first pre-treated with inhibitors for lysosome/endosomal acidification (chloroquine and bafilomycin A1), the recycling pathway (primaquine), cysteine proteases (leupeptin and E64), and acid proteases (pepstatin A) and then pulsed with polyepitope protein. Data presented in Figure 10A shows that rather than blocking the presentation of polyepitope proteins, lysosome, recycling pathway and cysteine protease inhibitors significantly increased the T cell recognition of CEM.T1 and/or CEM.T2 cells pulsed with the polyepitope protein. These observations suggest that the pre-treatment with leupeptin, E64 or pepstatin A may protect the $CD8^+$ T cell epitopes within the polyepitope protein from degradation by cysteine and acid proteases. Unexpectedly, chloroquine and bafilomycin A1 showed opposing effects on the cross-presentation of the polyepitope protein. While chloroquine enhanced the antigen presentation in CEM.T2 cells, pre-treatment with bafilomycin A1 significantly reduced the T cell recognition of polyepitope pulsed antigen presenting cells (Fig. 10A). Previous studies have shown that bafilomycin A1 is also a potent and specific inhibitor of vacuolar H+ ATPase and prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes. To explore whether the polyepitope protein processing may involve the autophagy pathway, we pre-treated antigen presenting cells with the PI3K inhibitor, 3-methyladenine (3-MA) and then exposed to CMV-specific T cells. Data

presented in Fig. 10A shows that 3-MA treatment also effected the presentation of $CD8^+$ T cell epitopes from the polyepitope protein. These observations suggest that it is likely that cross-presentation of the polyepitope protein is via an autophagy dependent pathway.

In the next set of experiments we investigated the potential role of the proteasome complex in cross-presentation of the polyepitope protein. CEM.T1 and CEM.T2 cells were pre-treated with the proteasome inhibitors lactacystin, epoxomicin and MG132 and then pulsed with polyepitope protein. These cells were then assessed for the presentation of $CD8^+$ T cell epitopes. Data presented in Figure 10B shows that all three proteasome inhibitors completely blocked the presentation of $CD8^+$ T cell epitopes from the polyepitope proteins. It is important to note that presentation of $CD8^+$ T cell epitopes does not depend on the expression of immunoproteasomes since CEM.T2 cells, which don't express these components of the proteolytic complex, can efficiently process $CD8^+$ epitopes from the polyepitope protein. We next focused our attention on the potential role of the secretory pathway and ER-resident aminopeptidases in the presentation of $CD8^+$ T cell epitopes from the polyepitope protein. Data presented in Figure 10C shows that pre-treatment with brefeldin-A and monensin significantly blocked presentation to $CD8^+$ T cells, while leucinethiol treatment had minimal effect on the T cell recognition of CEM.T1 and CEM.T2 cells. These results suggest that the polyepitope protein is processed via a proteasome dependent but ER independent pathway that may involve the retrotranslocation pathway, which degrades misfolded ER proteins.

To further elucidate the influence of retrotranslocation and autophagy mediated pathways in the cross-presentation of the polyepitope, CEM.T1 and CEM.T2 cells were infected with lentivirus expressing shRNAs for silencing of the Sec61β subunit and ATG12 (autophagy regulator 12) genes. The data presented in Figures 11A-C shows that although shRNA expression dramatically reduced the expression of Sec61β subunit, this loss of expression had minimal effect on the presentation of T cell epitopes from the polyepitope protein. In contrast, down-regulation of ATG12 expression in both CEM.T1 and CEM.T2 cells significantly reduced the recognition of CMV polyepitope protein sensitized cells. Taken together these observations demonstrate that cross-presentation of the polyepitope protein occurs through a novel pathway which involves both proteasomal and autophagy pathways.

EXAMPLE 2

Immunogenicity of CMV polyepitope protein in combination with adjuvants

Materials and Methods

CMV polyepitope vaccine formulation with MPL and CpG ODN1826

The CMV polyepitope vaccine was formulated by mixing 20 μg of CMVpoly, CMVpoly-PL or CMVpoly-PTL with 25 μg of MPL (TLR4 agonist) and 50 μg of CpG ODN1826 (TLR9 agonist) per dose in a 100 μL volume. TLR agonists were purchased from InvivoGen (San Diego, CA, USA).

Mouse immunisations

HHD I mice containing human HLA-A*0201 with a disrupted murine MHC class I were bred and maintained under specific pathogen-free conditions at the QIMR. All protocols were followed in compliance with the QIMR animal ethics committee. In each group at least 5 (M1-5), six-to-eight week old mice, were immunised subcutaneously (s.c.) at the base of the tail with the CMV polyepitope vaccine formulated with the above specified adjuvant combinations. Mice were boosted with an identical vaccine formulation on day 21 and mice were sacrified on day35 to determine the polyepitope specific $CD8^+$ T cell responses using intracellular cytokine staining (ICS) assay.

Splenocyte preparation

Mice were sacrificed by CO_2 asphyxiation and spleens were collected in 3 mL of mouse T cell culture medium (DMEM supplemented with 10% FBS, 100 IU/mL penicillin, 200 μg/mL streptomycin sulphate, β-mercaptoethanol, non-essential amino acids and sodium pyruvate). Single cell suspensions were prepared by gently mashing the spleen with a plunger of a syringe. Cells were centrifuged at 1200 rpm for 5 minutes, resuspended in 3 mL of ammonium chloride and Tris buffer (0.017M Tris base in 0.89% ammonium chloride, pH7.4) then incubated for five minutes at room temperature to deplete red blood cells. Cells were centrifuged, washed twice with PBS containing 2% FBS and resuspended in 5 mL of mouse T cell culture medium. To remove excess tissue and cellular debris, the final cell suspension was filtered through a 70 μm cell strainer (Becton Dickinson, San Diego, USA). Cell viability was then determined using the Trypan Blue exclusion method.

In vitro *stimulation and expansion of CMV specific T-cells from immunised mice*

Approximately $5x10^6$ splenocytes from vaccinated mice were stimulated with 1 μg of HLA A2 restricted NLV and VLE peptides in 100 μl of mouse T cell culture medium at 37°C, 6.5% CO_2 for 2 hours. After incubation, 1 mL of mouse T cell culture medium was added, cells were transferred to 24 well plate and cultured for 10 days at 37°C, 6.5% CO_2. On days 3 and 6, cultures were supplemented with 1 mL of T cell culture medium containing 100 U of recombinant IL-2. The T cell specificity of these in vitro expanded cells was assessed using a standard IFN-γ ICS assay. In addition, T cells in these cultures were also assessed for polyfunctional capacity using multi-parameter flow cytometery.

Intracellular cytokine staining to assess IFN-γ response in mouse T cells

Following *in vitro* stimulation with NLV and VLE approximately $2x10^5$ mouse splenocytes in 50 μL of mouse T cell culture medium were added to the required wells. To stimulate these cells 0.2 μg of NLV and VLE peptides were added and then 150 μL of DMEM containing 0.3 μL of Brefeldin A (BD Pharmingen, San Diego, CA) was added to each well and incubated for four hours at 37°C, 10% CO_2. Cells were washed twice with PBS containing 2% FCS (wash buffer), surface stained with APC-conjugated anti-CD3, FITC-conjugated anti-CD4 and PerCP-Cy5.5 conjugated anti-CD8 monoclonal antibodies resuspended in wash buffer and incubated at 4°C for 30 minutes. Cells were washed twice with wash buffer, fixed with 100 μL/ well of Cytofix/Cytoperm and washed twice with Perm/Wash buffer. Cells were then intracellularly stained with PE conjugated anti-IFN-γ monoclonal antibody at 4°C for 30 minutes, cells were washed twice Perm/Wash buffer and acquired on a BD FACSCanto II.

Multi-parametric flow cytometry to assess the immune responses in vaccinated mice

Following vaccination splenocytes were stimulated *ex vivo* as mentioned above. Cells were surface stained with FITC conjugated anti-CD4 and PerCP-Cy5.5 conjugated anti-CD8 for 30mins at 4° C. After washing, fixing and permeabilising, cells were stained intracellularly with PE-conjugated anti-IFN-γ, PE-Cy7 conjugated anti-TNF and APC conjugated anti-IL2 antibodies. Cells were acquired on a BD FACSCanto II and data was analysed using FlowJo software and Boolean gate analysis.

Results

In initial studies, a subunit vaccine formulation based on CMV-encoded glycoprotein B (gB) and polyepitope proteins was tested in combination with human compatible TLR agonists. The polyepitope protein included multiple minimal HLA class I-restricted $CD8^+$ T cell epitopes from different antigens of CMV. This subunit vaccine generated durable anti-viral antibody, Th1 $CD4^+$ and $CD8^+$ T cell responses. The humoral immune response induced by the vaccine displayed strong neutralisation capacity and the antigen-specific T cells expressed multiple cytokines with long-term memory maintenance. Furthermore, this subunit CMV vaccine, through the activation of TLR4 and TLR9, activated different dendritic cell (DC) subsets expressing IL12p70, IFN-α, IL-6 and TNF-α, which play a crucial role in the activation of antigen-specific T cells.

In vivo evaluation of immunogenicity of CMV polyepitope proteins with and without linkers

To determine the immunogenicity of CMVpoly, CMVpoly-PL and CMVpoly-PTL, we next evaluated the immunogenicity of the polyepitope proteins in combination with the TLR4 and TLR9 agonists. HHD-I transgenic mice expressing human HLA A2 MHC Class I allele were immunized with CMVpoly, CMVpoly-PL or CMVpoly-PTL. Following vaccination splenocytes were *in vitro* stimulated with HLA A2 restricted NLV and VLE peptides. To analyse the establishment CMV polyepitope-specific responses in *in vitro* stimulated splenocytes, they were assessed for the presence of CMVpoly-specific (HLA A2-restricted epitopes NLV and VLE) $CD8^+$ T cells using an intracellular IFN-γ assay. Interestingly, in line with *in vitro* data, mice immunised with CMVpoly-PL or CMVpoly-PTL vaccine formulation induced significantly higher frequencies of CMVpolyepitope-specific $CD8^+$ T cells compared to mice immunised with CMVpoly vaccine formulation (Figure 12A). In addition there is substantial evidence that the protective efficacy of T cell-based vaccines correlates with the frequencies of multifunctional effectors. Therefore in the subsequent experiments we assessed the functional quality of the CMV-specific $CD8^+$ T cell response. The pattern of IL-2, TNF and IFN-γ production was determined using multiparametric flow cytometry following *in vitro* expansion of splenocytes from immunised mice. Data presented in the Figure 12B clearly demonstrate that $CD8^+$ T cells displayed higher polyfunctionality; most importantly higher frequencies of $CD8^+$ T cells were IFN-γ and TNF producers in mice immunized with CMVpoly-PL or CMVpoly-PTL compared to CMVpoly vaccine. Taken together these observations clearly demonstrated

that a CMV vaccine formulation based on CMVpoly-PL or CMVpoly-PTL adjuvanted with both TLR4 and TLR9 agonist was most effective in inducing CMV-specific $CD8^+$ T cells with a multifunctional capability.

Discussion

Emerging evidence suggests that CMV-specific $CD8^+$ T cells responses in healthy CMV-seropositive individuals, are directed towards multiple CMV antigens, predominantly pp65 and IE1, but also other structural, early/late antigens and immunomodulators (pp28, pp50, pp150, IE2 gH, gB, US2, US3, US6 and UL18) (Elkington, Walker et al. 2003; Elkington, Shoukry et al. 2004; Manley, Luy et al. 2004; Khan, Bruton et al. 2005; Sylwester, Mitchell et al. 2005). These $CD8^+$ T cell responses play a critical role in immunity to CMV, controlling viral replication and preventing the clinical manifestations of progressive infection in both animal models as well as in humans (Quinnan, Kirmani et al. 1982; Rook, Quinnan et al. 1984; Reddehase, Weiland et al. 1985). These observations indicate that a vaccine against CMV that can induce T cells responses against multiple antigens will likely strengthen protection against CMV-associated disease. Therefore to target multiple antigens, especially to induce $CD8^+$ T cell responses, in this study we have proposed a novel recombinant based polyepitope vaccine technology. Polyepitope based vaccines provide a powerful approach to induce immune responses against a variety of conserved epitopes from a number of antigens without the use of full length antigens which may comprise unknown or pathogenic properties.

A series of CMV polyepitope proteins (13mer, 14mer, 15mer and 20mer) were designed by covalently linking multiple HLA class I restricted T-cell epitopes to potentiate CMV-specific $CD8^+$ T cell responses against a number of antigens in different ethnic populations. Selected epitopes in the CMV polyepitope constructs were derived from highly conserved multiple antigens of CMV, including pp65, pp50, pp150, DNAse, and IE-1 (Brytting, Wahlberg et al. 1992; Retiere, Imbert et al. 1998; Solache, Morgan et al. 1999). To enhance the immunogenicity of the CMV polyepitope, the selected $CD8^+$ T cell epitopes were linked together with a linker sequence consisting of a proteasome liberation amino acid sequence (AD or K or R) and a TAP (transporter associated with antigen processing) recognition motif (RIW, RQW, NIW or NQY) at the carboxyl terminus of each epitope. In this regard, published data shows that the use of the amino acid residues to provide proteasomal processing of the polyepitope proteins (Ishioka, Fikes et al. 1999; Kuttler,

Nussbaum et al. 2000; Livingston, Newman et al. 2001) and the motifs for TAP recognition are necessary for transporting the proteasome generated peptides into the endoplasmic reticulum (ER) (Uebel, Wiesmuller et al. 1999; Bazhan, Karpenko et al. 2010). The 13mer, 14mer and 15mer CMV polyepitope proteins were successfully expressed as recombinant proteins in *E.coli* and purified using Ni-NTA chromatography. However, our attempts to make the CMV polyepitope 20mer were unsuccessful due to its highly hydrophobic nature. The optimised protein expression conditions and purification protocol were consistent. Approximately 2 L of shaker flask culture yielded a substantial quantity of polyepitope proteins.

Next we tested the CMV polyepitope proteins immunogenicity in *in vitro* experiments by stimulating the healthy donor PBMC to augment the frequencies of CMV epitope specific $CD8^+$ T cells. The data from these studies clearly demonstrated that these CMV polyepitope proteins are highly efficient in generating CMV-specific $CD8^+$ T cells responses in virus healthy carriers. Interestingly, our results showed the feasibility of simultaneously amplifying multiple CMV peptide-specific $CD8^+$ T cell responses and these expended $CD8^+$ T cells demonstrated strong expression of IFN-γ, TNF, MIP-1β and CD107a by CMV-specific $CD8^+$ T cells following stimulation with polyepitope protein. These functional characteristics of the T cells are highly important for predicting the efficacy of T cell mediated immune responses and virus clearance [reviewed in (Seder, Darrah et al. 2008)]. In addition to expanding virus-specific $CD8^+$ T cells from healthy donors, we also tested the immunogenicity of the polyepitope protein using human B cells (LCLs) and epithelial cells (HEK293). In this context, the majority of the HLA restricted epitopes encoded by the CMV polyepitope were processed and presented efficiently to antigen-specific T cells, confirming the propensity of the polyepitope protein to deliver epitopes for presentation via the MHC class I pathway.

Although many studies have shown how exogenous proteins are internalised, processed and presented by MHC class I molecules on antigen presenting cells, exogenously loaded polyepitope protein processing and presentation by antigen presenting cells has never been reported. In general, cross-presentation of exogenous antigens by dendritic cells has been shown to operate using three different pathways. The first proposed model uses an indirect pathway of transferring exogenous antigens from phago-endosomes to the cytosol for proteasome dependent processing. Processed peptides are then loaded in the endoplasmic reticulum by the classical MHC class I machinery (Huang, Bruce et al.

1996). The second model is a direct, proteasome independent pathway whereby antigens are processed and loaded on MHC class I entirely in endosomal compartments (Shen, Sigal et al. 2004).

The third proposed model utilises the delivery of endoplasmic reticulum components to endocytic organelles or the transport of incoming antigen to the endoplasmic reticulum (Guermonprez, Saveanu et al. 2003; Houde, Bertholet et al. 2003). Indeed, in the development of effective vaccines, Immunotherapies against cancers as well as in immune tolerance to self antigens to prevent autoimmunity, cross-presentation of exogenous antigens to naïve $CD8^+$ T cells is the prerequisite for the induction of cytotoxic T cell responses (Rock and Shen 2005). We therefore elucidated the pathway by which CMV polyepitope was processed and cross-presented by CEM.T1 and CEM.T2 cells in the presence of various chemical inhibitors involved in different stages of antigen presentation.

Our results clearly demonstrate that the polyepitope is degraded into peptides in a TAP-independent, proteasome and autophagy-dependent pathway. Both CEM.T1 and CEM.T2 cells treated with proteasome inhibitors and autophagy inhibitors prevented effective presentation of $CD8^+$ T cells epitopes, while presentation was enhanced with lysosome, recycling pathway, cysteine proteases, acid proteases and ER-resident amino peptidases inhibitors. In addition, we also observed reduced presentation of $CD8^+$ T cells epitope by CEM.T1 and CEM.T2 following treatment with brefeldin A and monensin. This effect could be an indirect effect on presentation of $CD8^+$ T cell epitopes because these inhibitors are known to block the transport of newly synthesised MHC I molecules on to the cell surface.

Because processing and presentation of $CD8^+$ T cell epitopes was blocked by proteasome but not ER inhibitors, we hypothesised that $CD8^+$ T cell epitope presentation was mediated via a retrotranslocation pathway whereby exogenously antigens are internalised into phagosomes, then delivered into cytosol through a Sec61 channel and degraded by proteasome into oligopeptides before being transferred to MHC class I molecules in the ER (Ackerman, Giodini et al. 2006; Rock 2006). However, knock down of the Sec61β subunit protein in CEM.T1 and CEM.T2 had no effect on presentation of $CD8^+$ T cells epitopes, indicating that the retrotranslocation pathway may not involve in the processing and presentation of the polyepitope encoded $CD8^+$ T cell epitopes.

Although we found no evidence for the retrotranslocation pathway in the processing of the polyepitope proteins, we did found evidence for a role for the autophagy pathway

following knockdown of ATG12. ATG12 is an ubiquitin-like modifier and its covalent conjugation with another autophagy regulator, ATG5, and plays an essential role in autophagy formation and elongation (Mizushima, Noda et al. 1998; Mizushima, Sugita et al. 1998). Therefore, we conclude that $CD8^+$ T cell epitopes from the polyepitope protein are processed and presented by CEM.T1 and CEM.T2 cells through a novel TAP-independent, proteasome and autophagy dependent pathways.

This pathway is difficult to reconcile with the previously proposed cross-presentation models, however, documented evidence suggest that the collaboration between the proteasome and autophagy pathways is essential for protein quality control in the cell (Ding, Ni et al. 2007). In addition, although a proteasome and autophagy dependent pathway has never been reported in the context of cross-presentaion it has been show to be involved in the degradation of endogenously over expressed proteins (Webb, Ravikumar et al. 2003).

Thus, based on these observations we speculate that the polyepitope protein is processed and presented through a novel proteasome and autophagy dependent pathway. In summary, polyepitope proteins can be expressed as a recombinant proteins using prokaryotic expression system in a stable form. These polyepitope proteins are highly immunogenic and may have the preferential access to proteasome and autophagosome dependent pathway while cross-presentation by antigen presenting cells.

EXAMPLE 3

Immunogenicity of EBV polyepitope protein in combination with adjuvants

Materials and Methods

Construction of EBV polyepitope construct

An EBV polyepitope was designed to encode multiple HLA class I restricted T-cell epitopes from 9 different antigens (BMLF1, BRLF1, BZLF1, LMP2, LMP2a, EBNA1, EBNA3A, EBNA3B and EBNA3C). The epitope HLA restriction, amino acid sequences and amino acid locations of these epitopes are shown in Table 3 and illustrated schemtically in Figure 13A.

The polyepitope sequence was designed in such a way that each epitope sequence was preceded by a proteasome liberation amino acid sequence (AD or K or R) and a hexa-histidine tag was inserted at the c-terminus of each polyepitope protein to allow purification

using a nickel-nitrilotriacetic acid (Ni-NTA) column. The amino acid sequence of each construct was translated into DNA sequence based on *E. coli* codon utilisation and inserts were synthetically constructed (DNA2.0, California, USA) and cloned into an expression plasmid (pJexpress 404) under an isopropyl –β-D-thiogalactopyraniside (IPTG) inducible promoter. Synthetically designed EBV polyepitope was transformed into chemically competent *E. coli* DH5α (Invitrogen, Carlsbad, CA, USA) and plasmids were purified using a QIAGEN maxi prep kit (QIAGEN, Hilden, Germany).

Protein expression

Chemically competent *E. coli* BL21 (DE3) pLysS (Invitrogen, California, USA) was transformed with the EBV polyepitope expression vector. Transformed cells were plated on Luria Bertani (LB) agar supplemented with 100 μg/mL of ampicillin (LB-Amp) and plates were incubated overnight at 37°C. An isolated colony was picked and inoculated into 10 ml of LB-Amp broth and grown in a shaker at 37°C and 200 rpm overnight. A small amount of overnight culture was inoculated into 50 mL of LB-Amp broth and grown for 12 hours, then 1% of culture was transferred into 2 L of LB-Amp broth that was then was grown until the O.D. reached 0.6 at 600nm. EBV polyepitope protein induction was carried out by adding 1 mM/mL of IPTG. These cells were allowed to grow for an additional 4 hours and protein expression levels were determined by analysing un-induced and induced samples on 15% SDS-PAGE.

EBV polyepitope protein purification

At the end of the induction phase, *E. coli* culture was harvested by centrifugation at 10,000 rpm for 15 minutes, the cell pellet was resuspended in 80 mL of lysis buffer (25 mM Tris pH 7.4, 0.5% TritonX100, 150 mM NaCl, 0.5 mg/mL lysozyme) supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany) and incubated on the ice for 30 minutes. Cell lysis was carried out by sonication on ice for 4 x 5 minutes cycles with a 10 minute break between each cycle. The lysate was centrifuged at 13,000 rpm for 30 minutes and supernatant and pellet fractions were analysed using SDS-PAGE. Since the majority of the protein was found in the pellet fractions in the form of inclusion bodies (IBs), IBs were washed once with lysis buffer (without lysozyme) under stirring for two hours at RT and solubilised in 150 mL of solubilisation buffer (100 mM NaH_2PO_4, 10 mM Tris, 8 M urea, 0.5% TritonX100 pH 8.0) overnight at 4°C. The soluble protein was clarified by

centrifugation at 13,000 rpm for 30 minutes and supernatant was used for purification of polyepitope proteins.

To purify the EBV polyepitope protein we used 5 mL of Ni-NTA (QIAGEN, Hilden, Germany) metal-affinity chromatography matrix. The matrix was washed with 5 column volumes of distilled water followed by equilibration with 3 column volumes of solubilisation buffer. The soluble protein was loaded on the column and the flow rate was adjusted to 1 mL/minute. The unbound protein and impurities were washed-out with 10 column volumes of wash buffer 1 (100 mM NaH_2PO_4, 10 mM Tris, 8 M urea pH 6.3) and 20 column volumes of wash buffer 2 (100 mM NaH_2PO_4, 10 mM Tris, 8 M urea pH 5.9). The bound protein was eluted with elution buffer (100 mM NaH2PO4, 10 mM Tris, 8 M urea pH 4.3) and the eluted fractions were analysed using SDS-PAGE as shown in Figure 13B. The positive fractions were pooled together and purified EBV polyepitope protein was dialysed against 25 mM MES buffer at pH 3.5. Following dialysis, the EBV polyepitope protein was concentrated using Ultracel-10K spin columns (Millipore, County Cork, Ireland) followed by sterile filtration using 0.22μ membrane filter. Final EBV polyepitope protein estimation was carried out using a Bradford assay kit (Bio-Rad, Hercules, California, USA).

In vitro stimulation and expansion of EBV specific T-cells from healthy donors using polyepitope proteins

Peripheral blood mononuclear cells (PBMC) from healthy virus carriers were incubated with 25 μg of purified EBV polyepitope protein at 37°C, 6.5% CO_2 for 2 hours. After incubation, these PBMC were mixed with un-pulsed PBMC and resuspended in RPMI 1640 medium supplemented with 10% FCS (referred to as growth medium). These cells were cultured in a 24 well plate for 14 days at 37°C, 6.5% CO_2. On days 3, 6 and 9 cultures were supplemented with 1 mL of growth medium containing 100 U of recombinant IL-2. The T cell specificity of these *in vitro* expanded cells was assessed using a standard ICS assay.

Results

Design and Purification of EBV polyepitope proteinwith proteasome linkers

Having developed a well established protocol to design, express and purify recombinant polyepitope proteins for immunotherapy against CMV, in the subsequent studies we

extended such approaches to design another recombinant polyepitope protein for immunotherapy to combat EBV associated malignancies. In particular, for the treatment of EBV-associated relapsed Hodgkin disease and nasopharyngeal carcinoma EBV-specific $CD8^+$ T cells are considered to be more effective. However, generation of EBV-specific $CD8^+$ T cells is restricted by a number of limitations, such as complex manufacturing process and most often such process requires infectious clinical grade virus material, for instance, recombinant adenovirus vectors for delivering the antigens to antigen-presenting cells. Therefore to overcome such problems, we have designed a novel EBV polyepitope, which can be expressed using a bacterial expression system. An EBV polyepitope encoding 20 minimal $CD8^+$ T cell epitopes from 9 different antigens (BMLF1, LMP2a, BRLF1, LMP2, EBNA3A, BZLF1, EBNA3C, EBNA1 andEBNA3B) was designed as outlined in Figure 13A and each epitope in the polyepitope sequence was separated by a proteasome linker. A comprehensive list of EBV epitopes included in each of these polyepitope sequences are presented in Table 3. The EBV polyepitope construct was transformed into *E. coli*. Protein expression conditions was optimised and expressed protein was purified using Ni-NTA matrix. In line with CMV polyepitope proteins results, data obtained from these experiments showed that EBV polyepitope can be successfully expressed using a bacterial expression system and protein can be purified to homogeneity (Figure 13B).

In vitro evaluation of immunogenicity of CMV polyepitope proteins with and without linkers

To determine the potential efficacy of EBV polyepitope to expand the EBV-specific $CD8^+$ T cells, PBMC from a number of different donors were stimulated with purified recombinant EBV polyepitope protein. Following stimulation expanded EBV-specific $CD8^+$ T cells were assessed using intracellular cytokine assay. Data presented in Figure 14 shows that a considerable proportion of donors showed expansion of EBV-specific $CD8^+$ T cells following stimulation with EBV polyepitope compared to unstimulated PBMC. Interestingly, the cells from each donor recognised multiple epitopes restricted through a number of HLA class I alleles and the majority of the donors had higher frequencies of expanded $CD8^+$ T cells towards at least 3 different epitopes.

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Table 1: List of HLA class-1 restricted Epitopes included in the CMV polyepitope 13, 14 , 15 &20mer

HLA restriction	CMV antigen	SEQ ID NO	Sequence	Location	Code	13mer	14mer	15mer	20mer
HLA A1	pp50	11	VTEHDTLLY	245-253	VTE	+	+	+	+
HLA A2	pp65	12	NLVPMVATV	495-503	NLV	+	+	+	+
	IE-1	13	VLEETSVML	316-324	VLE	+	+	+	+
HLA A3	IE-1	8	KLGGALQAK	184-192	KLG	+			+
	pp150	14	TTVYPPSSTAK	945-955	TTV		+	+	
HLA 11	pp65	2	GPISHGHVLK	16-24	GPI	+	+	+	+
HLA A23/24	pp65	15	AYAQKIFKIL	248-257	AYA		+	+	+
HLA A24	pp65	3	QYDPVAALF	341-349	QYD	+	+	+	+
HLA B7	pp65	5	TPRVTGGGAM	417-426	TPR	+	+	+	+
	pp65	9	RPHERNGFTVL	265-275	RPH	+	+	+	+
HLA B8	IE-1	6	QIKVRVDMV	88-96	QIK	+	+	+	+
	IE-1	10	ELRRKMMYM	199-207	ELR	+	+	+	+
HLA B27	DNAse	16	ARVYEIKCR	274-282	ARV				+
HLA B35	pp65	1	FPTKDVAL	188-195	FPT	+	+	+	+
	pp65	7	IPSINVHHY	123-131	IPS	+	+	+	+
	pp65	17	CPSQEPMSIYVY	103-114	CPS				+
HLA B40/60	pp65	18	CEDVPSGKL	232-240	CED				+
HLA B41	gB	19	YAYIYTTYL	153-161	YAY				+
HLA B44	pp65	4	QEFFWDANDIY	511-521	QEFF	+	+	+	+
HLA B57	pp65	20	QAIRETVEL	331-339	QAI				+
HLA cw6	pp65	21	TRATKMQVI	211-219	TRA			+	+

Table 2

1	**FPTKDVALADRIW**	B35	pp65
2	**GPISGHVLKADNQY**	A11	pp65
3	**QYDPVAALFADRQW**	A24	pp65
4	**QEFFWDANDIYADRIW**	B44/DRw52	pp65
5	**TPRVTGGGAMRNIW**	B7	pp65
6	**QIKVRVDMVRNQY**	B8	IE-1
7	**IPSINVHHYRNQY**	B35	pp65
8	**KLGGALQAKADRIW**	A3	pp65
9	**RPHERNGFTVRNIW**	B7	pp65
10	**ELRRKMMYMADNIW**	B8	IE-1
11	**VTEHDTLLYKRQW**	A1	pp50
12	**NLVPMVATVKRQW**	A2	pp65
13	**VLEETSVMLKNIW**	A2	IE-1

*CTL epitope sequence is in **bold**, TAP sequence is in *italics* and proteasome processing sequence is underlined. SEQ ID NOS refer to bolded epitope sequence.

Table 3

HLA Restriction	EBV antigens	SEQ ID no	Sequence	Amino acid location	Abbreviated code
HLA A2	BMLF1	22	GLCTLVAML	280-288	GLC
	LMP2a	23	CLGGLLTMV	426-434	CLG
HLA A3	BRLF1	24	RVRAYTYSK	148-156	RVR
HLA A11	BRLF1	25	ATIGTAMYK	134-142	ATI
	LMP2a	26	SSCSSCPLSKI	340-350	SSC
HLA A23	LMP2	27	PYLFWLAAI	131-139	PYL
HLA A24	LMP2a	28	TYGPVFMCL	419-427	TYG
HLA A30	EBNA3A	29	AYSSWMYSY	176-184	AYS
HLA B7	EBNA 3A	30	RPPIFIRRL	379-387	RPP
HLA B8	EBNA3A	31	FLRGRAYGL	325-333	FLR
	BZLF1	32	RAKFKQLL	190-197	RAK
HLA B15	EBNA3C	33	QNGALAINTF	213-222	QNG
HLA B27	EBNA3C	34	RRIYDLIEL	258-266	RRI
HLA B35/B*5301	EBNA1	35	HPVGEADYFEY	407-417	HPV
	BZLF1	36	LPEPLPQGQLTAY	52-64	LPE
HLA B40	LMP2a	37	IEDPPFNSL	200-208	IED
HLA B44	EBNA3C	38	EENLLDFVRFMGV	281-293	EEN
	EBNA3B	39	VEITPYKPTW	657-666	VEI
	BZLF1	40	EECDSELEIKRY	169-180	EEC
HLA B57/B58	EBNA3B	41	VSFIEFVGW	279-287	VSF

Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. It will therefore be appreciated by those of skill in the art that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments exemplified without departing from the scope of the present invention.

All computer programs, algorithms, patent and scientific literature referred to herein is incorporated herein by reference in their entirety.

CLAIMS

1. An isolated protein comprising respective amino acid sequences of each of a plurality of CTL epitopes from two or more different herpesvirus antigens and which further comprises an intervening amino acid or amino acid sequence between at least two of said CTL epitopes comprising proteasome liberation amino acids or amino acid sequences and, optionally, Transporter Associated with Antigen Processing (TAP) recognition motifs, wherein the isolated protein is capable of eliciting a cytotoxic T-lymphocyte immune response upon administration to an animal as an exogenous protein.

2. The isolated protein of Claim 1, wherein the epitopes are restricted by the HLA class I specificities HLA-Al, -A2, -A3, -All, -A23, -A24, -A26, -A29, -A30, -B7, -B8, -B27, - B35, -B38, -B40, -B41, -B44, -B51, -B57 and/or -B58.

3. The isolated protein of Claim 1, wherein the herpesvirus is cytomegalovirus (CMV) or Epstein-Barr virus (EBV).

4. The isolated protein of Claim 3, wherein the CMV CTL epitopes are derived from pp50, pp65, pp 150 and/or IE-l.

5. The isolated protein of Claim 4, wherein the CTL epitopes have an amino acid sequence selected from the group consisting of the amino acid sequences set forth in SEQ ID NOS: 1-21.

6. The isolated protein of Claim 5, which comprises each of the CTL epitope amino acid sequences set forth in SEQ ID NOS: 1-13.

7. The isolated protein of Claim 5 or Claim 6, which comprises a CTL epitope amino acid sequence set forth in SEQ ID NO:11.

8. The isolated protein of any preceding claim which comprises an amino acid sequence selected from the group consisting of: SEQ ID NO:42; SEQ ID NO:43: SEQ ID NO:44; SEQ ID NO:45: SEQ ID NO:46: SEQ ID NO:47; and SEQ ID NO:48.

9. The isolated protein of Claim 3, wherein the EBV CTL epitopes are derived from one or more antigens selected from the group consisting of: BMLF1, LMP2a, BRLF1, LMP2, EBNA3A, BZLF1, EBNA3C, EBNA1 and EBNA3B.

10. The isolated protein of Claim 8, wherein the CTL epitopes have an amino acid sequence selected from the group consisting of the amino acid sequences set forth in SEQ ID NOS: 22-41.

11. The isolated protein of any one of Claims 1-3, Claim 9 or Claim 10, wherein the isolated protein comprises an amino acid sequence set forth in SEQ ID NO:49.

12. The isolated protein of any preceding claim which comprises less than twenty (20) CTL epitopes.

13. The isolated protein of Claim 10, which comprises ten (10), eleven (11), twelve (12), thirteen (13), fourteen (14), fifteen (15), sixteen (16), seventeen (17), eighteen (18) or nineteen (19) CTL epitopes.

14. The isolated protein of any preceding claim which comprises epitopes from the same or different herpesviruses.

15. The isolated protein of any preceding claim, wherein the proteasome liberation amino acids or amino acid sequences comprise AD, K and/or R.

16. An isolated nucleic acid encoding the isolated protein of any preceding claim.

17. The isolated nucleic acid of Claim 16, which comprises a nucleotide sequence selected from the group consisting of: SEQ ID NO:50; SEQ ID NO:51; SEQ ID NO:52: SEQ ID NO:53; SEQ ID NO:54; SEQ ID NO:55; SEQ ID NO:56; and SEQ ID NO:57.

18. A genetic construct comprising the isolated nucleic acid of Claim 16 or Claim 17 operably linked to one or more regulatory sequences in an expression vector.

19. A host cell comprising the genetic construct of Claim 18.

20. A method of producing an isolated protein according to any one of Claims 1-15, said method including step of at least partly purifying the isolated protein under conditions that maintain the isolated protein in a substantially non-aggregated form.

21. A pharmaceutical composition comprising: (i) one or more of the isolated proteins of any one of Claims 1-15; (ii) one or more of isolated proteins

produced by the method of Claim 20 and/or (iii) a genetic construct according to Claim 18; and a pharmaceutically-acceptable carrier, diluent or excipient.

22. The pharmaceutical composition of Claim 21, further comprising an immunostimulatory molecule or adjuvant.

23. The pharmaceutical composition of Claim 21, wherein the immunostimulatory molecule or adjuvant is TLR agonists that includes a TLR4 agonist and/or a TLR9 agonist.

24. The pharmaceutical composition of Claim 21, Claim 22 or Claim 23, which is a vaccine for eliciting a protective immune response against a herpesvirus in a human.

25. The pharmaceutical composition of any one of Claims 22-24, which is a vaccine for eliciting a protective immune response against CMV and/or EBV in a human.

26. A method of prophylactically or therapeutically treating a herpesvirus infection in an animal including the step of administering to the animal the isolated protein of any one of Claims 1-15 or the pharmaceutical composition of any one of Claims 21-25, to thereby prophylactically or therapeutically treat the herpesvirus infection in the animal.

27. A method of inducing or eliciting a cytotoxic T-lymphocyte (CTL) immune response in an animal including the step of administering to the animal the the isolated protein of any one of Claims 1-15 or the pharmaceutical composition of any one of Claims 21-25, to thereby induce or elicit a cytotoxic T-lymphocyte (CTL) immune response in said animal.

28. A method of expanding herpesvirus-specific CTLs for adoptive immunotherapy, including the steps of:

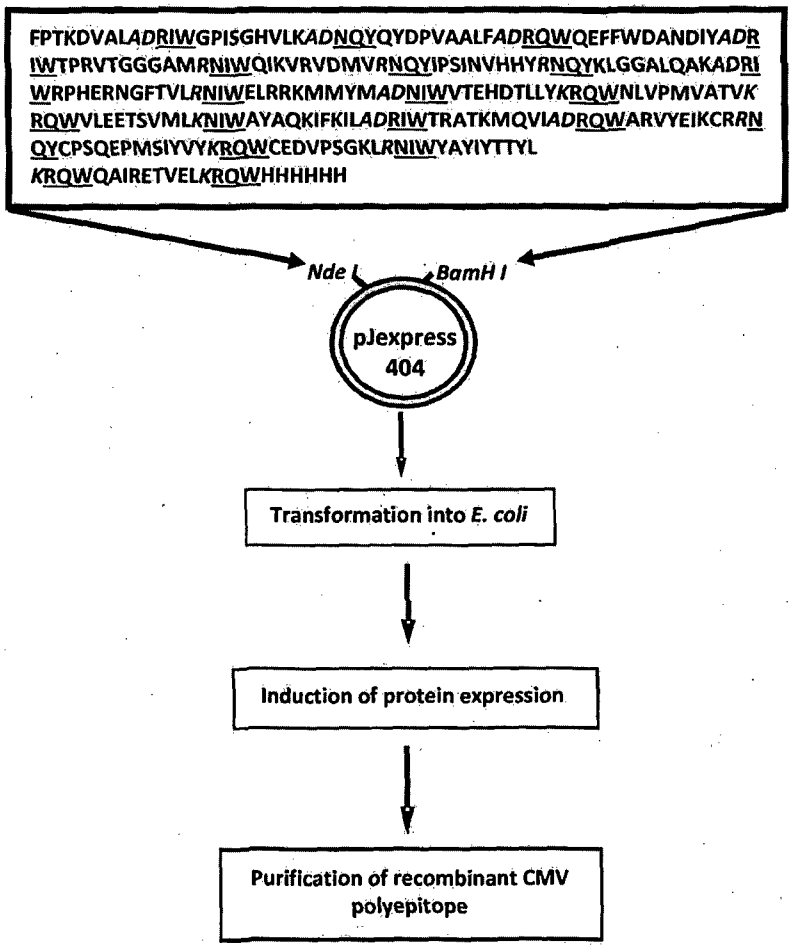
(a) contacting one or more cells isolated from an animal with the isolated protein of any one of Claims 1-15; and

(b) culturing said one or more cells to thereby expand herpesvirus-specific CTLs from said one or more cells.

29. A method of adoptive immunotherapy including the step of administering said CMV-specific CTLs produced at step (b) of Claim 28 to an animal to thereby prophylactically or therapeutically treat a CMV infection of said animal.

30. The method of any one of Claims 26-29 wherein the herpesvirus is CMV or EBV.

31. The method of any one of Claims 26-30 wherein the animal is a human.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IWTPRVTGGGAMRNIWQIKVRVDMVRNQYIPSINVHHYRNQYKLGGALQAKADRI
WRPHERNGFTVLRNIWELRRKMMYMADNIWVTEHDTLLYKRQWNLVPMVATVK
RQWVLEETSVMLKNIWAYAQKIFKILADRIWTRATKMQVIADRQWARVYEIKCRRN
QYCPSQEPMSIYVYKRQWCEDVPSGKLRNIWYAYIYTTYL
KRQWQAIRETVELKRQWHHHHHH
Nde I
BamH I
pJexpress 404
Transformation into E. coli
Induction of protein expression
Purification of recombinant CMV polyepitope

Figure 1

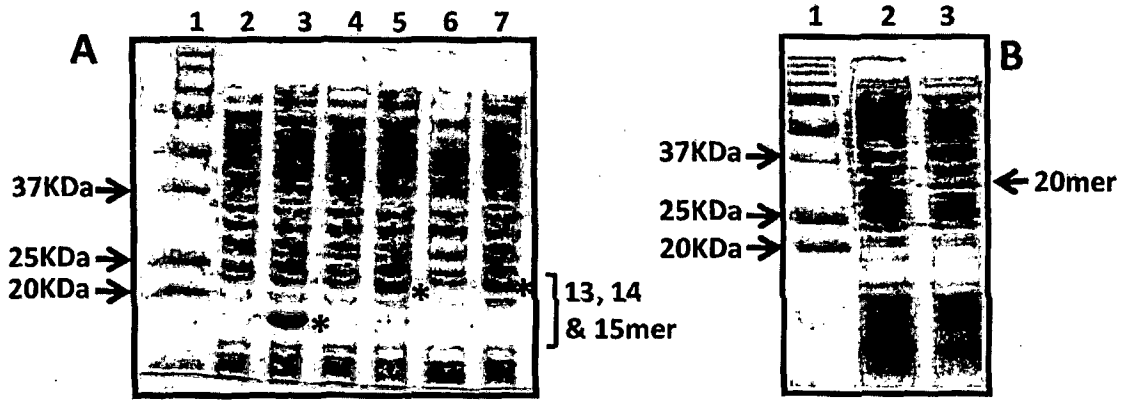

A
1 2 3 4 5 6 7
37KDa
25KDa
20KDa
13, 14
& 15mer
B
1 2 3
37KDa
25KDa
20KDa
20mer

Figure 2

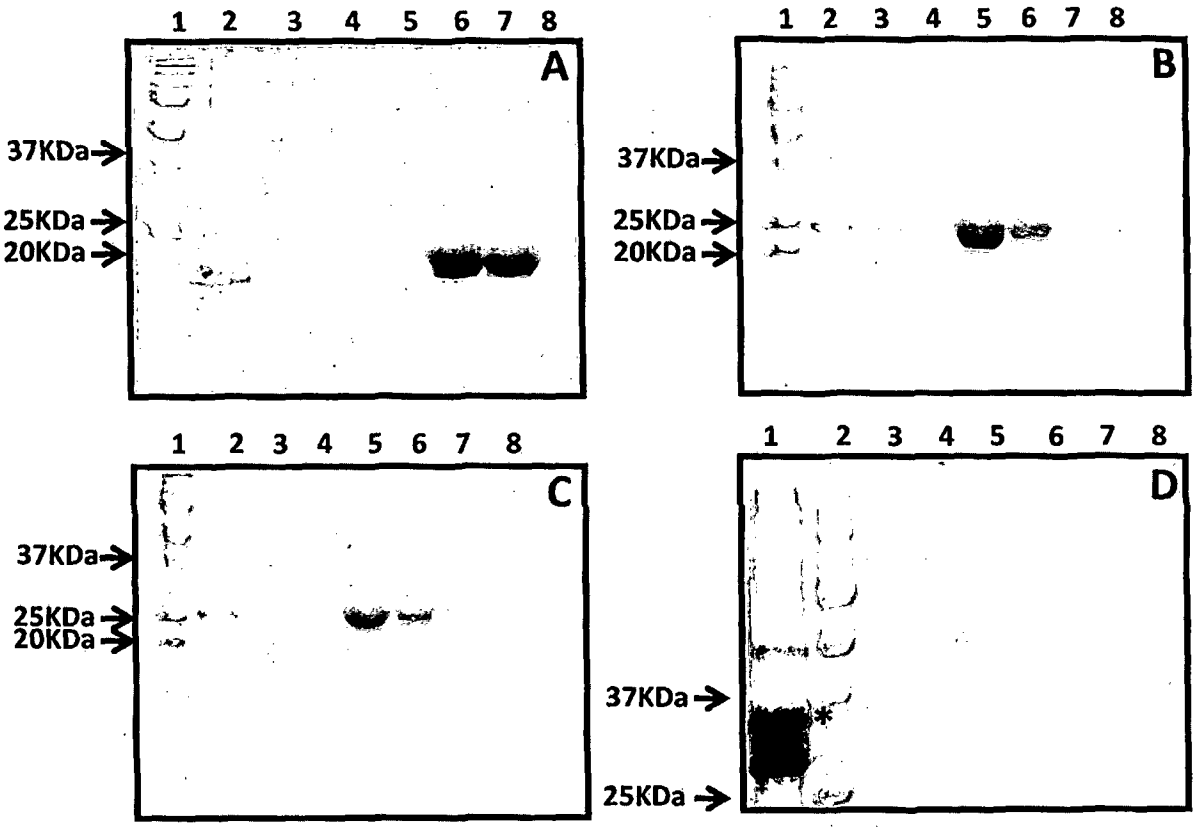

1 2 3 4 5 6 7 8
A
37KDa
25KDa
20KDa
1 2 3 4 5 6 7 8
B
37KDa
25KDa
20KDa
1 2 3 4 5 6 7 8
C
37KDa
25KDa
20KDa
1 2 3 4 5 6 7 8
D
37KDa
25KDa

Figure 3

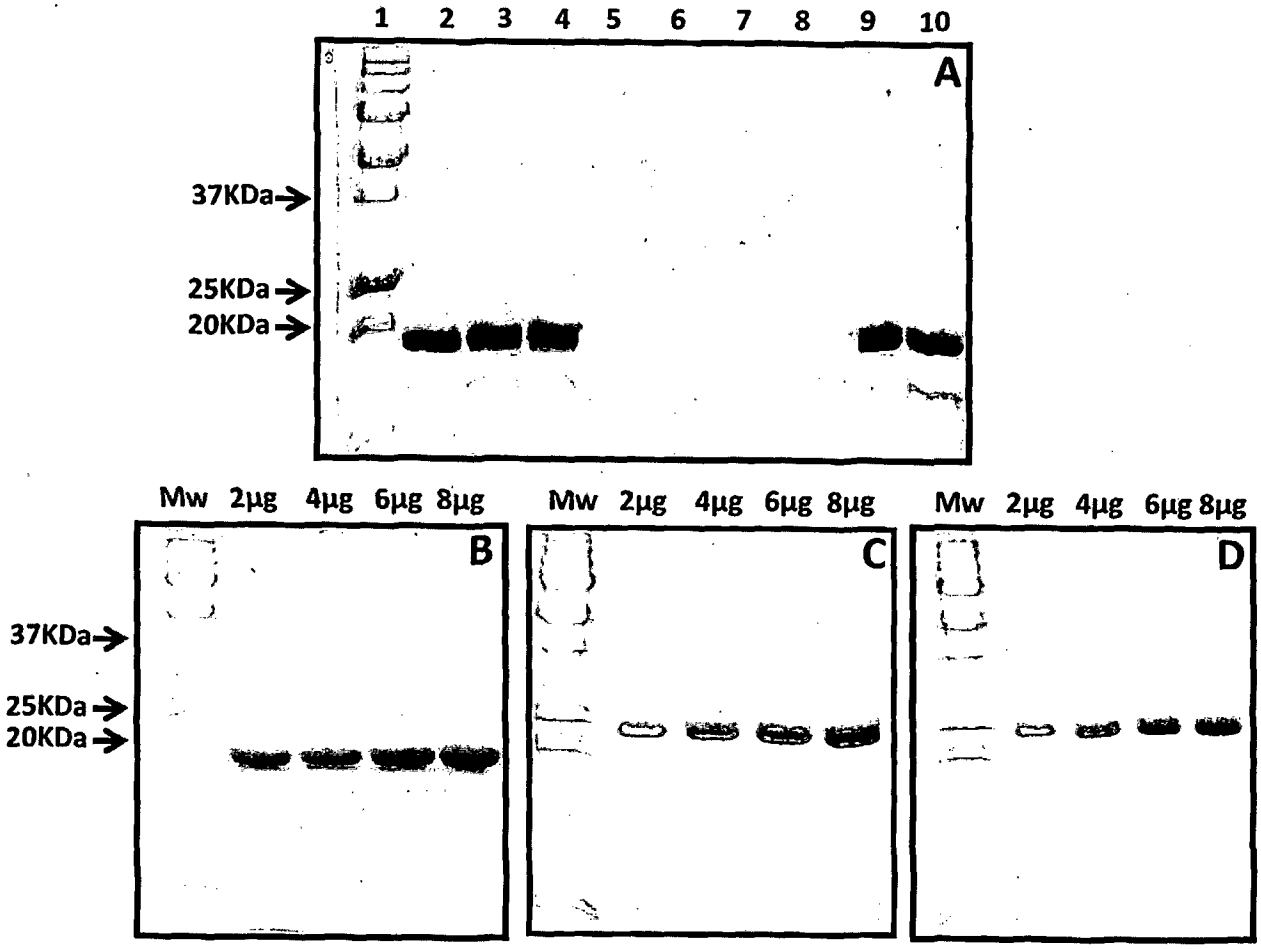

1 2 3 4 5 6 7 8 9 10
A
37KDa
25KDa
20KDa
Mw 2µg 4µg 6µg 8µg
B
Mw 2µg 4µg 6µg 8µg
C
Mw 2µg 4µg 6µg 8µg
D
37KDa
25KDa
20KDa

Figure 4

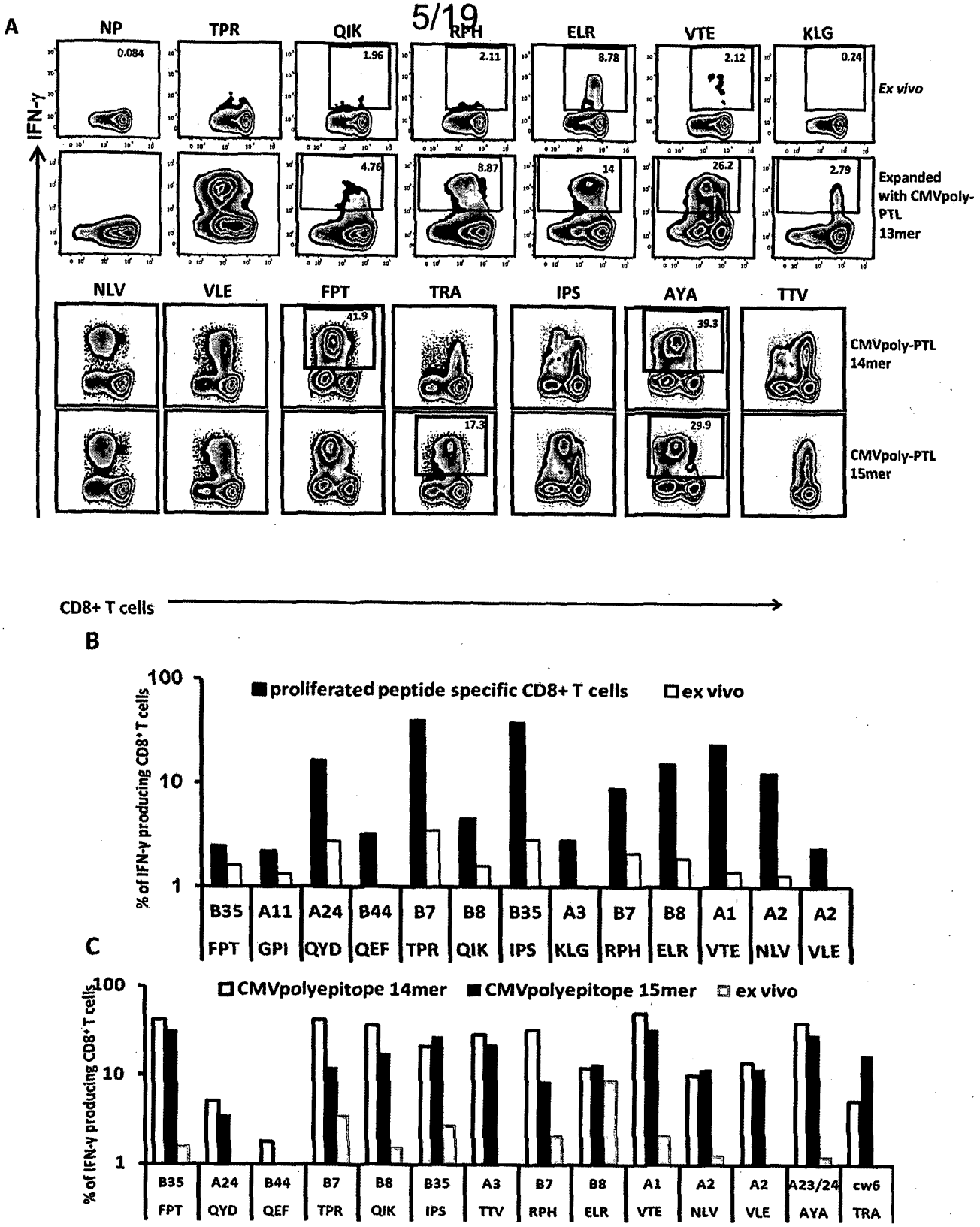

A
NP
TPR
QIK
RPH
ELR
VTE
KLG
IFN-γ
0.084
1.96
2.11
8.78
2.12
0.24
Ex vivo
4.76
8.87
14
26.2
2.79
Expanded with CMVpoly-PTL 13mer
NLV
VLE
FPT
TRA
IPS
AYA
TTV
41.9
39.3
17.3
29.9
CMVpoly-PTL 14mer
CMVpoly-PTL 15mer
CD8+ T cells
B
proliferated peptide specific CD8+ T cells
ex vivo
% of IFN-γ producing CD8+ T cells
100
10
1
B35 FPT
A11 GPI
A24 QYD
B44 QEF
B7 TPR
B8 QIK
B35 IPS
A3 KLG
B7 RPH
B8 ELR
A1 VTE
A2 NLV
A2 VLE
C
CMVpolyepitope 14mer
CMVpolyepitope 15mer
ex vivo
% of IFN-γ producing CD8+ T cells
100
10
1
B35 FPT
A24 QYD
B44 QEF
B7 TPR
B8 QIK
B35 IPS
A3 TTV
B7 RPH
B8 ELR
A1 VTE
A2 NLV
A2 VLE
A23/24 AYA
cw6 TRA

Figure 5

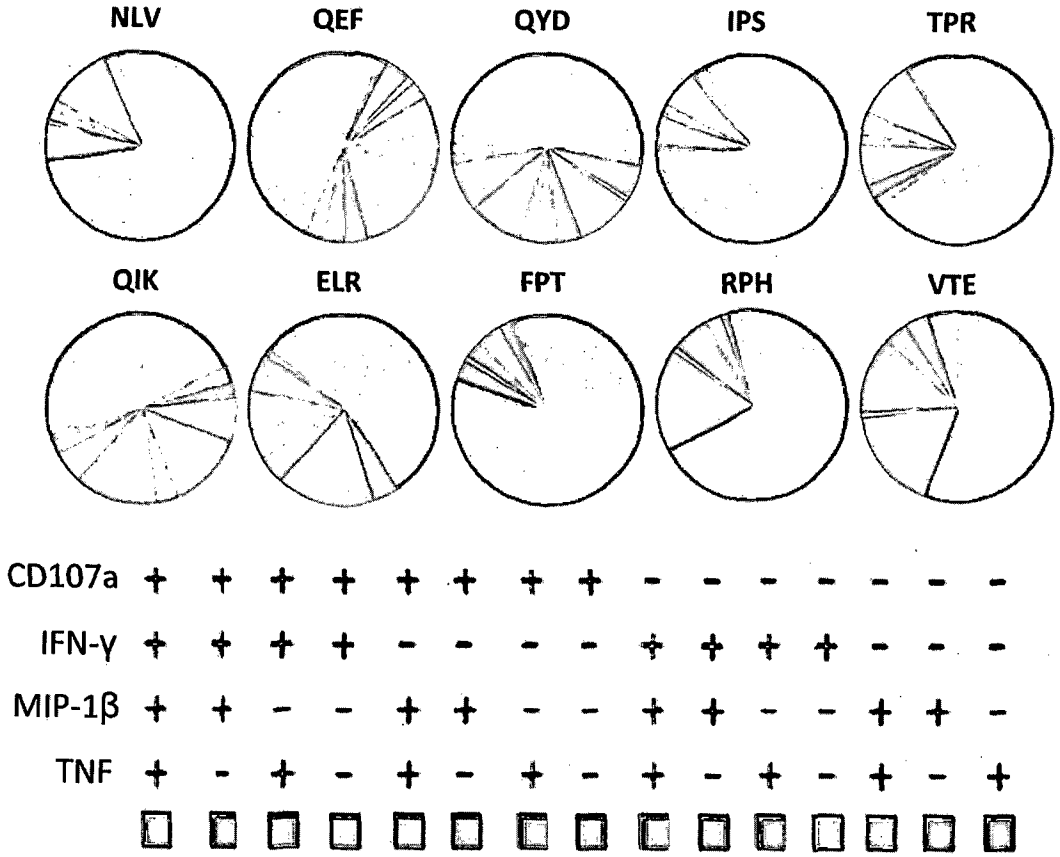
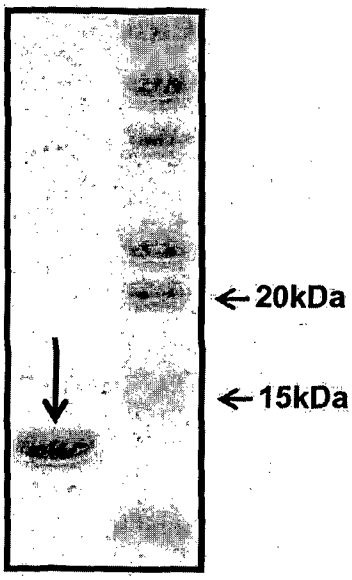


Figure 6

A

FPTKDVALGPISGHVLKQYDPVAALFQEFFWDANDIYTP
RVTGGGAMQIKVRVDMVIPSINVHHYKLGGALQAKRP
HERNGFTVLELRRKMMYMVTEHDTLLYNLVPMVATVV
LEETSVMHHHHHH



B

FPTKDVALADGPISGHVLKADQYDPVAALFADQEFFW
DANDIYADTPRVTGGGAMRQIKVRVDMVRIPSINVHH
YRKLGGALQAKADRPHERNGFTVLRELRRKMMYMAD
VTEHDTLLYKNLVPMVATVKVLEETSVMLKHHHHHH

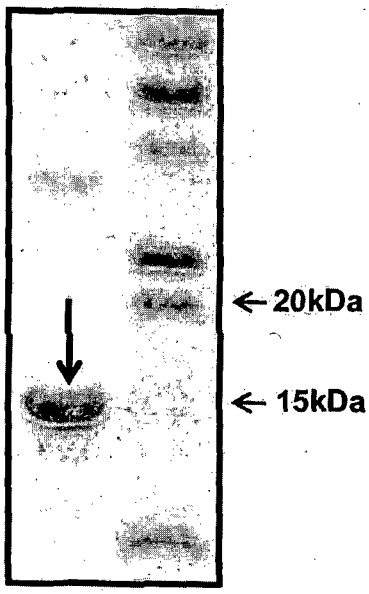


Figure 7

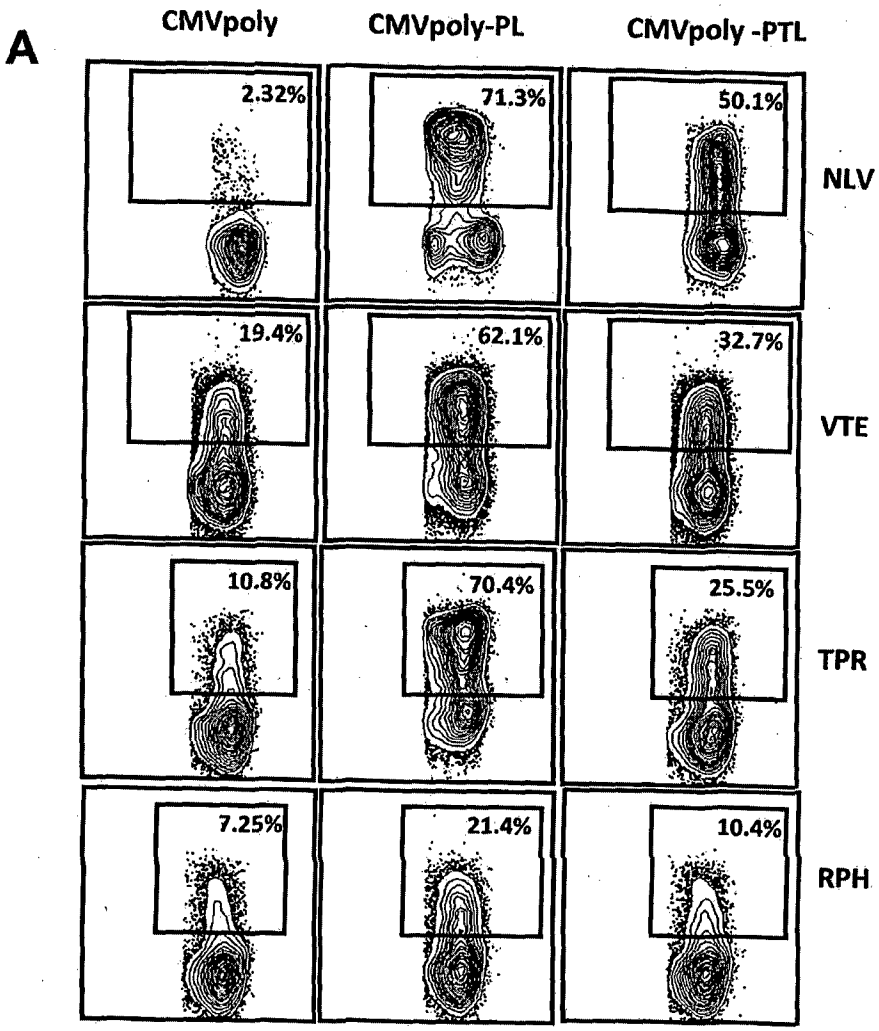

A
CMVpoly
CMVpoly-PL
CMVpoly -PTL
2.32%
71.3%
50.1%
NLV
19.4%
62.1%
32.7%
VTE
10.8%
70.4%
25.5%
TPR
7.25%
21.4%
10.4%
RPH
B
% of IFN-γ producing CD8+ T cells
0
10
20
30
40
50
60
70
80
NLV
VTE
ELR
TPR
RPH
CMVpoly
CMVpoly-PL
CMVpoly-PTL

Figure 8

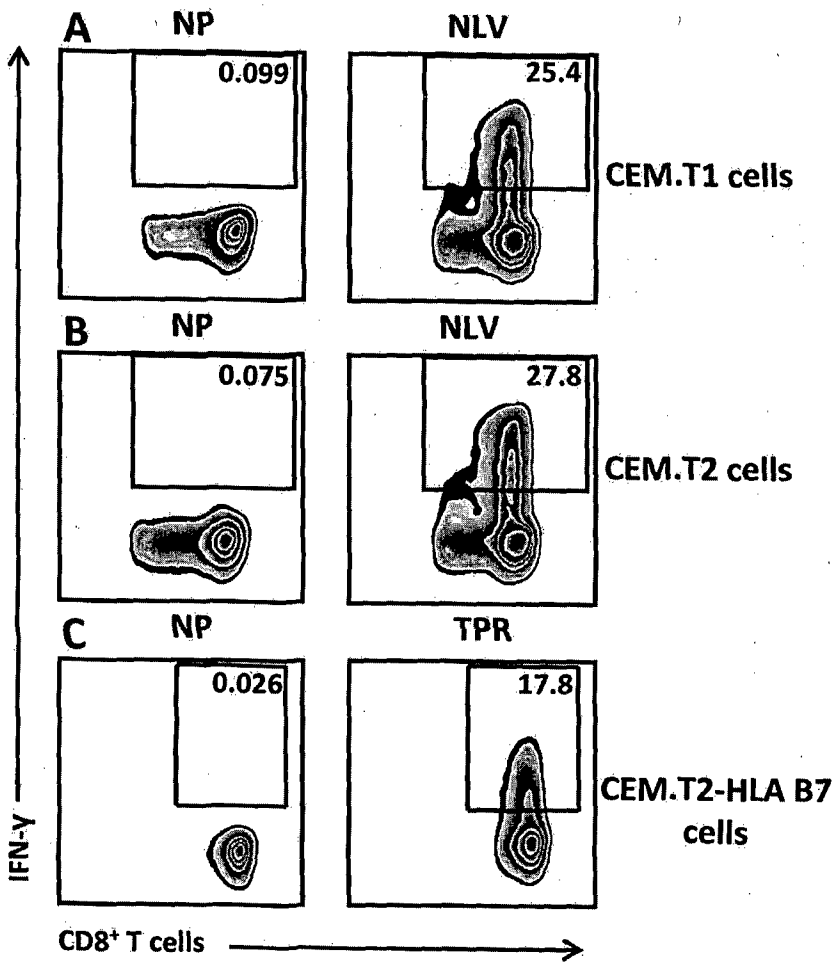

A
NP
NLV
0.099
25.4
CEM.T1 cells
B
NP
NLV
0.075
27.8
CEM.T2 cells
C
NP
TPR
0.026
17.8
CEM.T2-HLA B7 cells
IFN-γ
CD8+ T cells

Figure 9

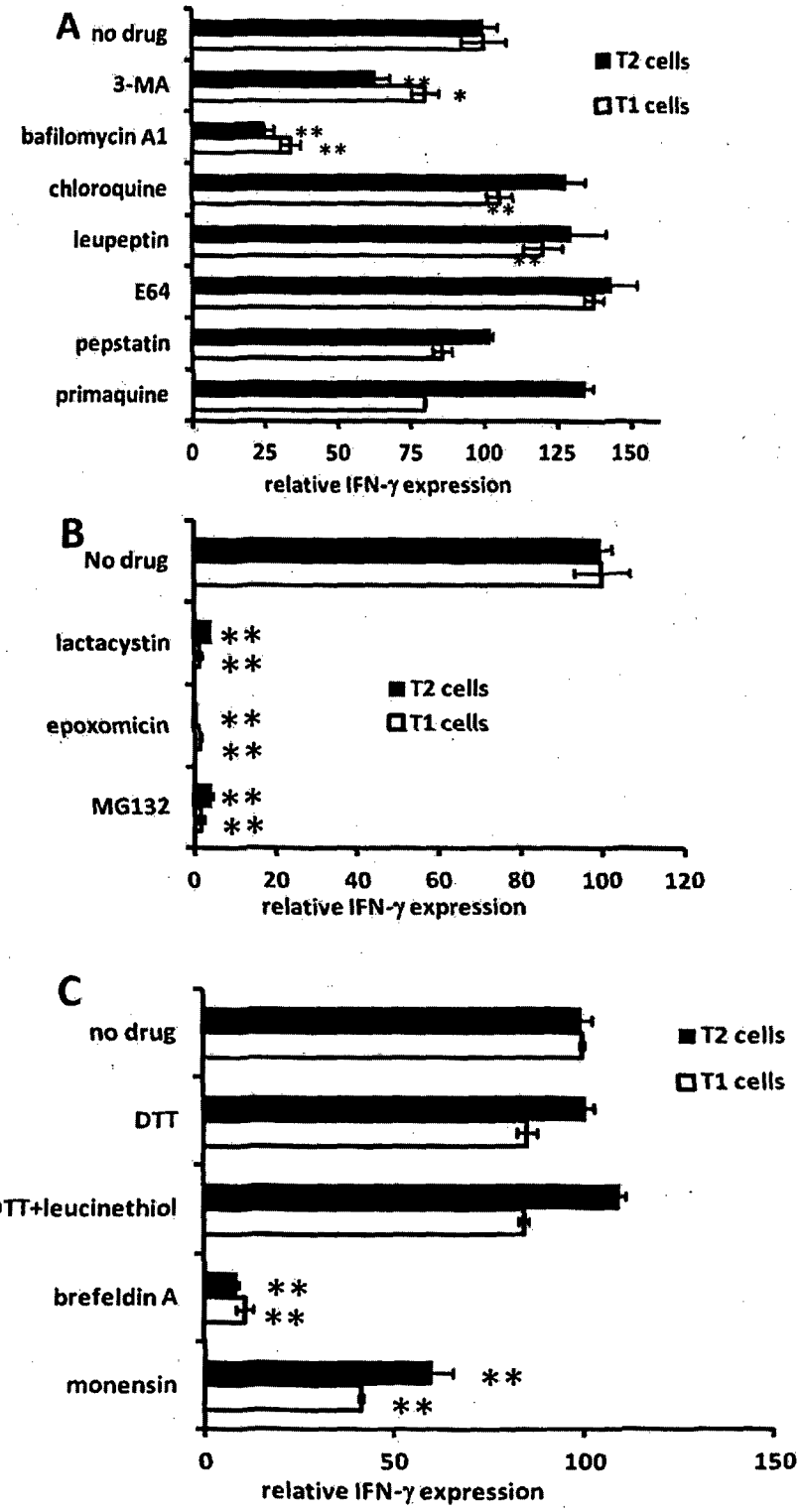

A
no drug
3-MA
bafilomycin A1
chloroquine
leupeptin
E64
pepstatin
primaquine
T2 cells
T1 cells
0 25 50 75 100 125 150
relative IFN-γ expression
B
No drug
lactacystin
epoxomicin
MG132
T2 cells
T1 cells
0 20 40 60 80 100 120
relative IFN-γ expression
C
no drug
DTT
DTT+leucinethiol
brefeldin A
monensin
T2 cells
T1 cells
0 50 100 150
relative IFN-γ expression

Figure 10

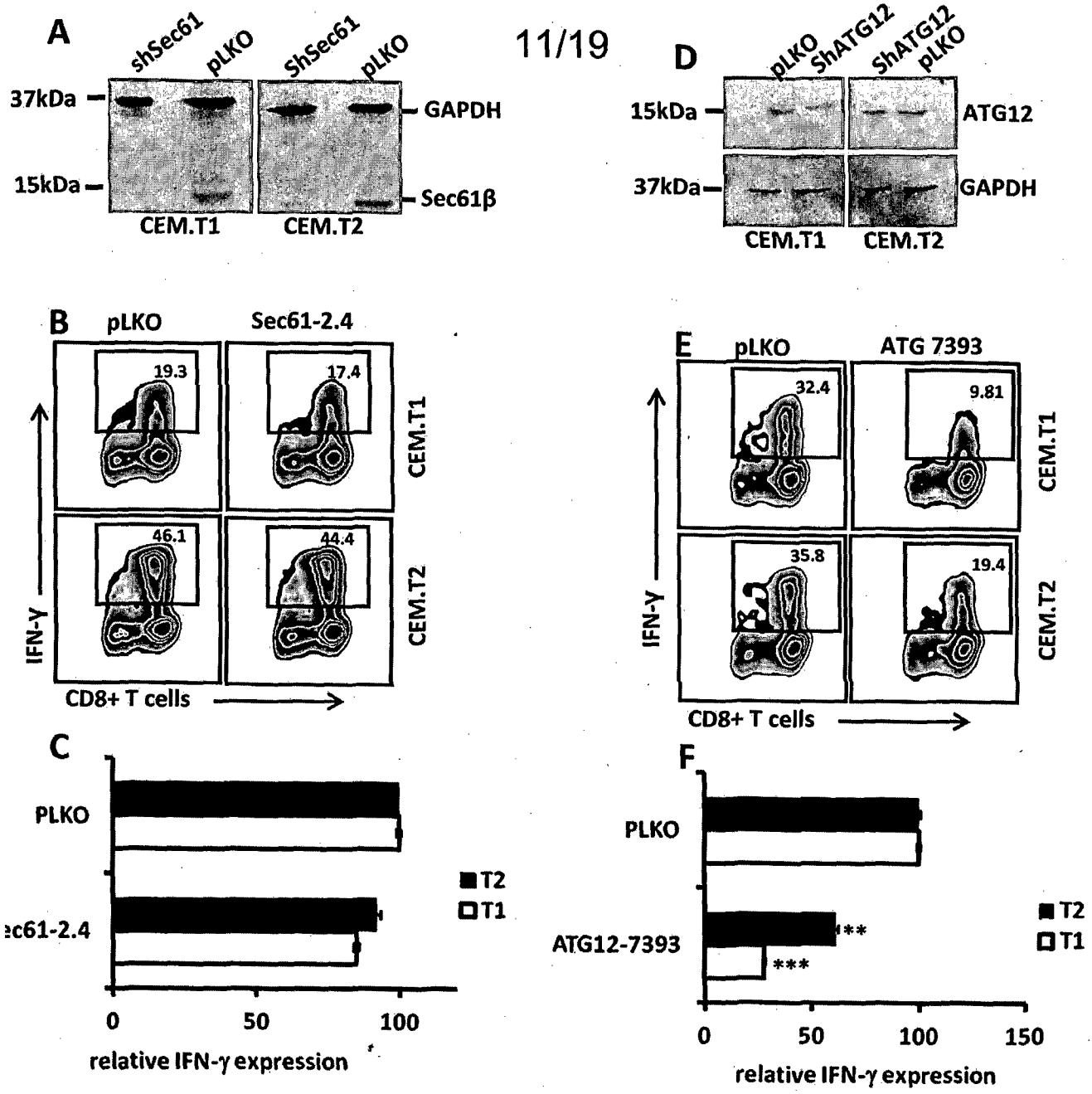

A
shSec61
pLKO
ShSec61
pLKO
37kDa
15kDa
GAPDH
Sec61β
CEM.T1
CEM.T2
D
pLKO
ShATG12
ShATG12
pLKO
15kDa
37kDa
ATG12
GAPDH
CEM.T1
CEM.T2
B
pLKO
Sec61-2.4
19.3
17.4
46.1
44.4
CEM.T1
CEM.T2
IFN-γ
CD8+ T cells
E
pLKO
ATG 7393
32.4
9.81
35.8
19.4
CEM.T1
CEM.T2
IFN-γ
CD8+ T cells
C
PLKO
ec61-2.4
T2
T1
0
50
100
relative IFN-γ expression
F
PLKO
ATG12-7393
**
***
T2
T1
0
50
100
150
relative IFN-γ expression

Figure 11

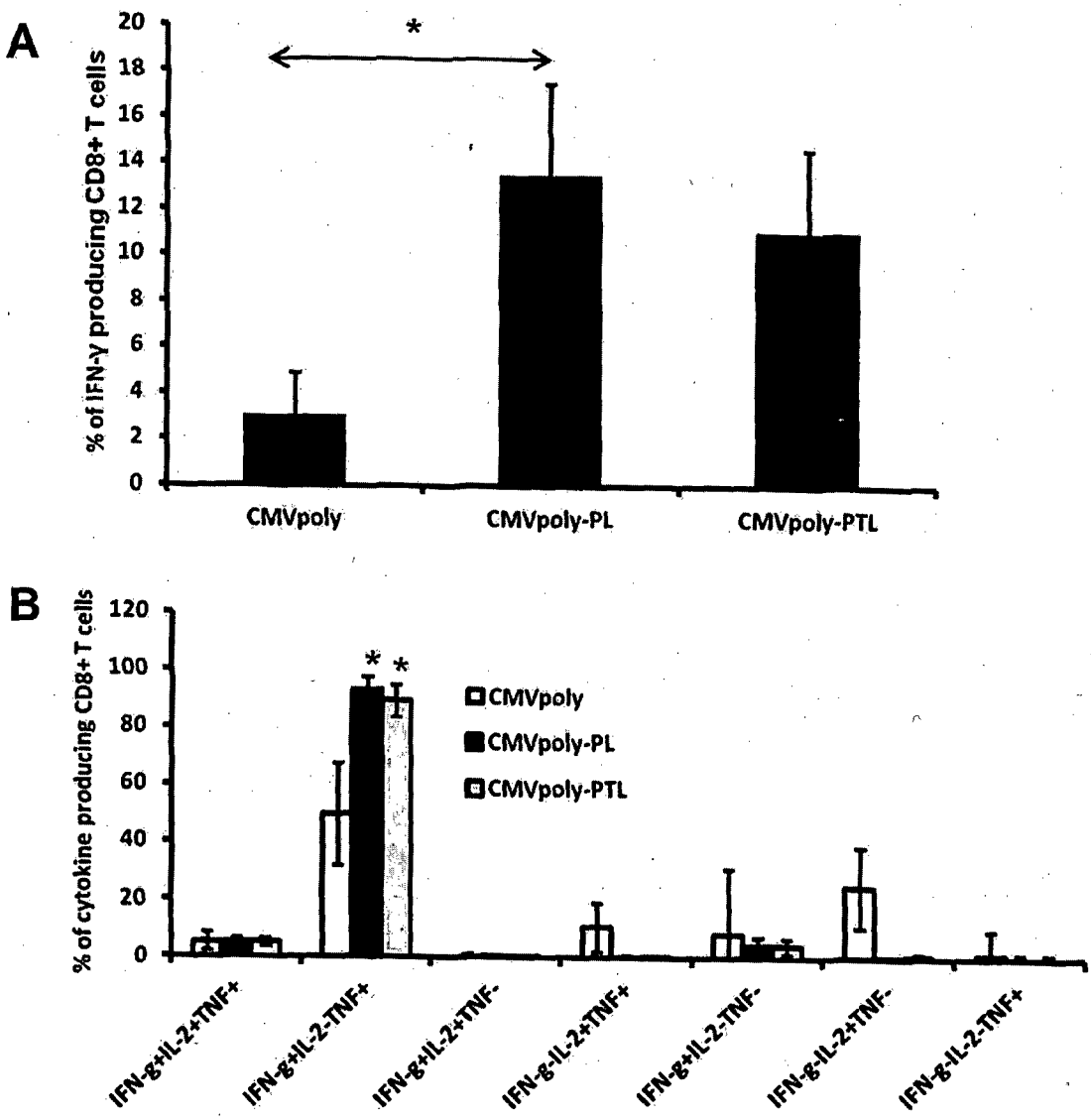

A
% of IFN-γ producing CD8+ T cells
20
18
16
14
12
10
8
6
4
2
0
*
CMVpoly
CMVpoly-PL
CMVpoly-PTL
B
% of cytokine producing CD8+ T cells
120
100
80
60
40
20
0
* *
CMVpoly
CMVpoly-PL
CMVpoly-PTL
IFN-g+IL-2+TNF+
IFN-g+IL-2-TNF+
IFN-g+IL-2+TNF-
IFN-g-IL-2+TNF+
IFN-g+IL-2-TNF-
IFN-g-IL-2+TNF-
IFN-g-IL-2-TNF+

Figure 12

A

HPVGEADYFEYRSSCSSCPLSKIADRPPIFIRRLKFLRGRAYGLRGLCTL
VAMLADEECDSELEIKRYKCLGGLLTMVADRAKFKQLLRATIGTAMY
KADTYGPVFMCLKLPEPLPQGQLTAYKIEDPPFNSLADVSFIEFVGW
KEENLLDFVRFMGVKQNGALAINTFRPYLFWLAAIRAYSSWMYSYA
DRVRAYTYSKADRRIYDLIELRVEITPYKPTWADHHHHHH

B

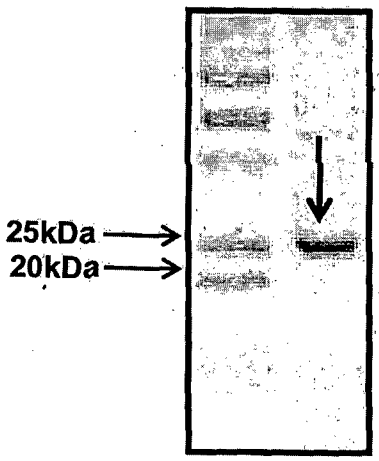


Figure 13

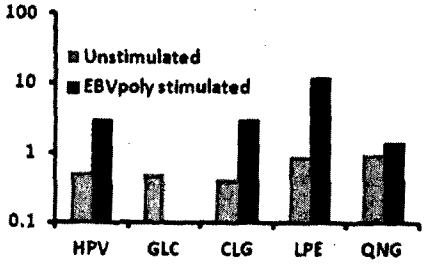


Fig. 14a

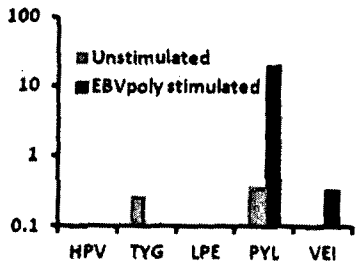


Fig. 14b

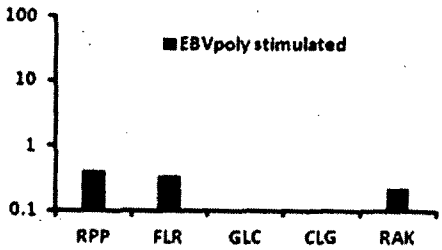


Fig. 14c

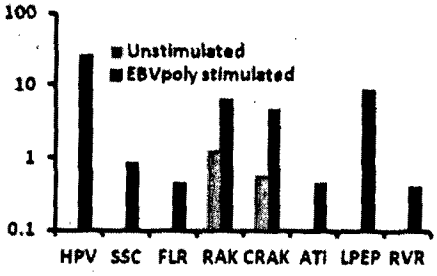


Fig. 14d

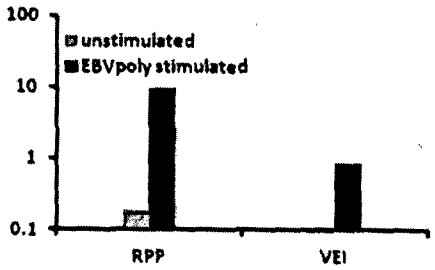


Fig. 14e

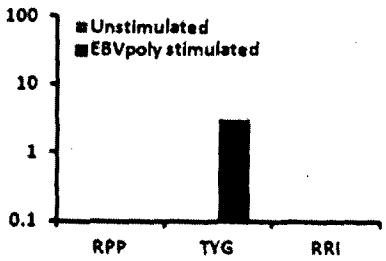


Fig. 14f

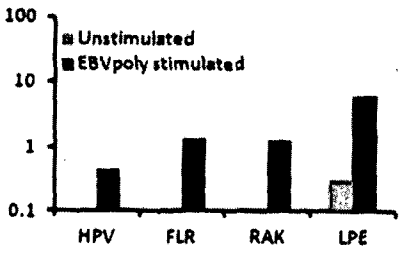


Fig. 14g

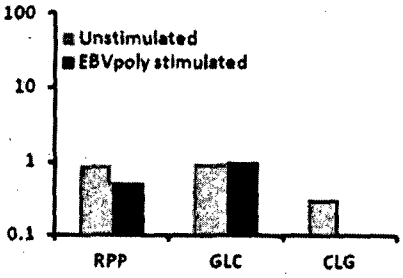


Fig. 14h

A CMVpoly with proteasome and TAP linkers with his tag

```
  1  M  F  P  T  K  D  V  A  L  A  D  R  I  W  G  P  I  S  H  G
  1 ATGTTCCCAACCAAAGACGTCGCACTCGCTGACCGCATCTGGGGCCCTATTAGCCATGGT
 21  H  V  L  K  A  D  N  Q  Y  Q  Y  D  P  V  A  A  L  F  A  D
 61 CACGTCCTGAAAGCGGACAATCAATATCAGTATGACCCGGTTGCCGCGTTGTTTGCGGAT
 41  R  Q  W  Q  E  F  F  W  D  A  N  D  I  Y  A  D  R  I  W  T
121 CGTCAATGGCAGGAGTTCTTCTGGGACGCTAACGACATCTACGCAGATCGTATCTGGACT
 61  P  R  V  T  G  G  G  A  M  R  N  I  W  Q  I  K  V  R  V  D
181 CCGCGCGTTACGGGCGGTGGCGCCATGCGCAACATTTGGCAGATCAAAGTCCGCGTGGAT
 81  M  V  R  N  Q  Y  I  P  S  I  N  V  H  H  Y  R  N  Q  Y  K
241 ATGGTACGCAATCAGTATATTCCGTCCATTAATGTTCACCACTACCGTAACCAGTACAAA
101  L  G  G  A  L  Q  A  K  A  D  R  I  W  R  P  H  E  R  N  G
301 CTGGGTGGTGCCCTGCAGGCGAAGGCGGATCGTATTTGGCGTCCGCACGAGCGCAATGGT
121  F  T  V  L  R  N  I  W  E  L  R  R  K  M  M  Y  M  A  D  N
361 TTTACGGTGCTGCGTAATATCTGGGAGCTGCGTCGTAAGATGATGTACATGGCGGATAAC
141  I  W  V  T  E  H  D  T  L  L  Y  K  R  Q  W  N  L  V  P  M
421 ATTTGGGTGACCGAACATGATACCCTGTTGTACAAACGTCAATGGAACCTGGTGCCGATG
161  V  A  T  V  K  R  Q  W  V  L  E  E  T  S  V  M  L  K  N  I
481 GTTGCAACGGTGAAGCGTCAATGGGTTCTGGAAGAAACCAGCGTCATGCTGAAGAACATC
181  W  H  H  H  H  H  H  *
541 TGGCATCATCATCACCACCACTAA
```

B CMVpoly with proteasome and TAP linkers with no his tag

```
  1  M  F  P  T  K  D  V  A  L  A  D  R  I  W  G  P  I  S  H  G
  1 ATGTTCCCAACCAAAGACGTCGCACTCGCTGACCGCATCTGGGGCCCTATTAGCCATGGT
 21  H  V  L  K  A  D  N  Q  Y  Q  Y  D  P  V  A  A  L  F  A  D
 61 CACGTCCTGAAAGCGGACAATCAATATCAGTATGACCCGGTTGCCGCGTTGTTTGCGGAT
 41  R  Q  W  Q  E  F  F  W  D  A  N  D  I  Y  A  D  R  I  W  T
121 CGTCAATGGCAGGAGTTCTTCTGGGACGCTAACGACATCTACGCAGATCGTATCTGGACT
 61  P  R  V  T  G  G  G  A  M  R  N  I  W  Q  I  K  V  R  V  D
181 CCGCGCGTTACGGGCGGTGGCGCCATGCGCAACATTTGGCAGATCAAAGTCCGCGTGGAT
 81  M  V  R  N  Q  Y  I  P  S  I  N  V  H  H  Y  R  N  Q  Y  K
241 ATGGTACGCAATCAGTATATTCCGTCCATTAATGTTCACCACTACCGTAACCAGTACAAA
101  L  G  G  A  L  Q  A  K  A  D  R  I  W  R  P  H  E  R  N  G
301 CTGGGTGGTGCCCTGCAGGCGAAGGCGGATCGTATTTGGCGTCCGCACGAGCGCAATGGT
121  F  T  V  L  R  N  I  W  E  L  R  R  K  M  M  Y  M  A  D  N
361 TTTACGGTGCTGCGTAATATCTGGGAGCTGCGTCGTAAGATGATGTACATGGCGGATAAC
141  I  W  V  T  E  H  D  T  L  L  Y  K  R  Q  W  N  L  V  P  M
421 ATTTGGGTGACCGAACATGATACCCTGTTGTACAAACGTCAATGGAACCTGGTGCCGATG
161  V  A  T  V  K  R  Q  W  V  L  E  E  T  S  V  M  L  K  N  I
481 GTTGCAACGGTGAAGCGTCAATGGGTTCTGGAAGAAACCAGCGTCATGCTGAAGAACATC
181  W  *
541 TGGTAA
```

Figure 15

C CMVpoly14mer

```
  1  M  F  P  T  K  D  V  A  L  A  D  R  I  W  G  P  I  S  H  G
  1 ATGTTTCCAACCAAAGACGTTGCACTCGCTGACCGCATCTGGGGCCCTATTTCTCACGGT
 21  H  V  L  K  A  D  N  Q  Y  Q  Y  D  P  V  A  A  L  F  A  D
 61 CATGTTCTGAAGGCCGATAACCAATATCAGTACGACCCGGTCGCGGCATTGTTCGCGGAC
 41  R  Q  W  Q  E  F  F  W  D  A  N  D  I  Y  A  D  R  I  W  T
121 CGCCAGTGGCAAGAGTTCTTTTGGGATGCCAACGATATCTATGCGGATCGTATTTGGACG
 61  P  R  V  T  G  G  G  A  M  R  N  I  W  Q  I  K  V  R  V  D
181 CCGCGTGTGACGGGTGGTGGCGCGATGCGTAACATCTGGCAAATCAAAGTGCGTGTCGAC
 81  M  V  R  N  Q  Y  I  P  S  I  N  V  H  H  Y  R  N  Q  Y  T
241 ATGGTGCGTAATCAGTATATTCCGAGCATTAACGTGCATCACTACCGCAATCAATATACC
101  T  V  Y  P  P  S  S  T  A  K  A  D  N  Q  Y  R  P  H  E  R
301 ACGGTCTACCCGCCGAGCAGCACCGCAAAAGCTGACAATCAGTATCGTCCGCATGAGCGC
121  N  G  F  T  V  L  R  N  I  W  E  L  R  R  K  M  M  Y  M  A
361 AATGGTTTTACCGTGCTGCGTAATATCTGGGAACTGCGTCGTAAAATGATGTACATGGCG
141  D  N  I  W  V  T  E  H  D  T  L  L  Y  K  R  Q  W  N  L  V
421 GACAACATCTGGGTCACGGAGCACGATACCCTGCTGTACAAGCGCCAGTGGAATCTGGTC
161  P  M  V  A  T  V  K  R  Q  W  V  L  E  E  T  S  V  M  L  K
481 CCGATGGTTGCGACCGTTAAACGCCAGTGGGTTCTGGAAGAAACTTCCGTTATGCTGAAA
181  N  I  W  A  Y  A  Q  K  I  F  K  I  L  K  R  Q  W  H  H  H
541 AACATTTGGGCATACGCCCAAAAGATTTTCAAGATCCTGAAGCGTCAATGGCACCATCAC
201  H  H  H  *
601 CACCACCATTAA
```

D CMVpoly15mer

```
  1  M  F  P  T  K  D  V  A  L  A  D  R  I  W  G  P  I  S  H  G
  1 ATGTTTCCAACCAAAGACGTTGCACTCGCTGACCGCATCTGGGGCCCTATTTCTCACGGT
 21  H  V  L  K  A  D  N  Q  Y  Q  Y  D  P  V  A  A  L  F  A  D
 61 CATGTTCTGAAGGCCGATAACCAATATCAGTACGACCCGGTCGCGGCATTGTTCGCGGAC
 41  R  Q  W  Q  E  F  F  W  D  A  N  D  I  Y  A  D  R  I  W  T
121 CGCCAGTGGCAAGAGTTCTTTTGGGATGCCAACGATATCTATGCGGATCGTATTTGGACG
 61  P  R  V  T  G  G  G  A  M  R  N  I  W  Q  I  K  V  R  V  D
181 CCGCGTGTGACGGGTGGTGGCGCGATGCGTAACATCTGGCAAATCAAAGTGCGTGTCGAC
 81  M  V  R  N  Q  Y  I  P  S  I  N  V  H  H  Y  R  N  Q  Y  T
241 ATGGTGCGTAATCAGTATATTCCGAGCATTAACGTGCATCACTACCGCAATCAATATACC
101  T  V  Y  P  P  S  S  T  A  K  A  D  N  Q  Y  R  P  H  E  R
301 ACGGTCTACCCGCCGAGCAGCACCGCAAAAGCTGACAATCAGTATCGTCCGCATGAGCGC
121  N  G  F  T  V  L  R  N  I  W  E  L  R  R  K  M  M  Y  M  A
361 AATGGTTTTACCGTGCTGCGTAATATCTGGGAACTGCGTCGTAAAATGATGTACATGGCG
141  D  N  I  W  V  T  E  H  D  T  L  L  Y  K  R  Q  W  N  L  V
421 GACAACATCTGGGTCACGGAGCACGATACCCTGCTGTACAAGCGCCAGTGGAATCTGGTC
161  P  M  V  A  T  V  K  R  Q  W  V  L  E  E  T  S  V  M  L  K
481 CCGATGGTTGCGACCGTTAAACGCCAGTGGGTTCTGGAAGAAACTTCCGTTATGCTGAAA
181  N  I  W  A  Y  A  Q  K  I  F  K  I  L  K  R  Q  W  T  R  A
541 AACATTTGGGCATACGCCCAAAAGATTTTCAAGATCCTGAAGCGTCAATGGACCCGTGCG
201  T  K  M  Q  V  I  A  D  R  I  W  H  H  H  H  H  H  *
601 ACCAAGATGCAGGTGATCGCGGATCGCATTTGGCACCATCACCACCACCATTAA
```

Figure 15

E **CMVpoly20mer**

```
  1  M  F  P  T  K  D  V  A  L  A  D  R  I  W  G  P  I  S  G  H
  1 ATGTTCCCGACTAAAGACGTTGCACTGGCCGACCGCATCTGGGGTCCGATTAGCGGTCAC
 21  V  L  K  A  D  N  Q  Y  Q  Y  D  P  V  A  A  L  F  A  D  R
 61 GTGCTGAAAGCAGACAACCAATACCAGTATGACCCGGTCGCAGCGCTGTTTGCGGATCGC
 41  Q  W  Q  E  F  F  W  D  A  N  D  I  Y  A  D  R  I  W  T  P
121 CAGTGGCAAGAGTTCTTTTGGGACGCAAATGACATTTATGCCGATCGCATCTGGACGCCT
 61  R  V  T  G  G  G  A  M  R  N  I  W  Q  I  K  V  R  V  D  M
181 CGTGTGACCGGTGGTGGCGCAATGCGTAATATCTGGCAGATTAAGGTGCGTGTGGATATG
 81  V  R  N  Q  Y  I  P  S  I  N  V  H  H  Y  R  N  Q  Y  K  L
241 GTGCGTAATCAGTATATTCCGAGCATCAATGTTCACCATTATCGTAATCAATACAAGCTG
101  G  G  A  L  Q  A  K  A  D  R  I  W  R  P  H  E  R  N  G  F
301 GGTGGCGCCCTGCAGGCTAAGGCAGATCGTATCTGGCGTCCGCATGAGCGTAACGGTTTT
121  T  V  R  N  I  W  E  L  R  R  K  M  M  Y  M  A  D  N  I  W
361 ACGGTCCGTAACATCTGGGAATTGCGTCGCAAAATGATGTATATGGCCGACAACATTTGG
141  V  T  E  H  D  T  L  L  Y  K  R  Q  W  N  L  V  P  M  V  A
421 GTTACCGAGCATGACACCCTGCTGTACAAACGCCAGTGGAATCTGGTGCCGATGGTTGCG
161  T  V  K  R  Q  W  V  L  E  E  T  S  V  M  L  K  N  I  W  A
481 ACGGTTAAGCGCCAATGGGTTCTGGAAGAAACCTCTGTCATGCTGAAGAATATCTGGGCG
181  Y  A  Q  K  I  F  K  I  L  A  D  R  I  W  T  R  A  T  K  M
541 TATGCCCAGAAGATTTTCAAGATTCTGGCCGATCGTATTTGGACGCGTGCAACCAAAATG
201  Q  V  I  A  D  R  Q  W  A  R  V  Y  E  I  K  C  R  R  N  Q
601 CAGGTCATTGCGGACCGTCAGTGGGCGCGTGTCTACGAAATCAAGTGCCGCCGTAACCAG
221  Y  C  P  S  Q  E  P  M  S  I  Y  V  Y  K  R  Q  W  C  E  D
661 TATTGTCCGAGCCAGGAGCCGATGAGCATCTACGTGTACAAGCGTCAGTGGTGTGAGGAC
241  V  P  S  G  K  L  R  N  I  W  Y  A  Y  I  Y  T  T  Y  L  K
721 GTTCCGAGCGGCAAGCTGCGCAATATCTGGTACGCCTACATCTACACCACCTATCTGAAA
261  R  Q  W  Q  A  I  R  E  T  V  E  L  K  R  Q  W  H  H  H  H
781 CGTCAATGGCAAGCGATTCGTGAAACCGTTGAGCTGAAAAGACAATGGCACCACCATCAC
281 H  H  *
841 CACCATTAA
```

Figure 15

F **CMVpoly with no linkers**

```
  1  M  F  P  T  K  D  V  A  L  G  P  I  S  H  G  H  V  L  K  Q
  1 ATGTTCCCAACTAAAGATGTAGCACTCGGTCCAATTTCGCACGGTCACGTTCTGAAGCAA
 21  Y  D  P  V  A  A  L  F  Q  E  F  F  W  D  A  N  D  I  Y  T
 61 TACGATCCGGTTGCCGCTCTGTTCCAGGAGTTCTTTTGGGACGCAAACGACATCTACACG
 41  P  R  V  T  G  G  G  A  M  Q  I  K  V  R  V  D  M  V  I  P
121 CCGCGTGTTACCGGCGGTGGCGCGATGCAGATCAAGGTGCGCGTGGATATGGTGATTCCG
 61  S  I  N  V  H  H  Y  K  L  G  G  A  L  Q  A  K  R  P  H  E
181 AGCATCAATGTGCACCACTATAAACTGGGTGGTGCGTTGCAAGCGAAACGTCCGCATGAG
 81  R  N  G  F  T  V  L  E  L  R  R  K  M  M  Y  M  V  T  E  H
241 CGTAACGGCTTTACGGTTCTGGAACTGCGTCGCAAGATGATGTACATGGTGACCGAGCAT
101  D  T  L  L  Y  N  L  V  P  M  V  A  T  V  V  L  E  E  T  S
301 GACACCCTGTTGTATAATCTGGTCCCGATGGTTGCGACCGTCGTCCTGGAAGAAACGAGC
121  V  M  L  H  H  H  H  H  H  *
361 GTCATGCTGCACCACCATCATCATCACTAA
```

G **CMVpoly with proteasome linkers**

```
  1  M  F  P  T  K  D  V  A  L  A  D  G  P  I  S  H  G  H  V  L
  1 ATGTTCCCAACTAAAGATGTCGCACTCGCAGATGGTCCAATTTCTCACGGTCACGTATTG
 21  K  A  D  Q  Y  D  P  V  A  A  L  F  A  D  Q  E  F  F  W  D
 61 AAGGCGGATCAGTACGACCCGGTTGCCGCTCTGTTTGCCGATCAAGAGTTCTTCTGGGAC
 41  A  N  D  I  Y  A  D  T  P  R  V  T  G  G  G  A  M  R  Q  I
121 GCTAACGATATCTATGCCGACACCCCGCGTGTGACGGGTGGTGGCGCAATGCGCCAAATC
 61  K  V  R  V  D  M  V  R  I  P  S  I  N  V  H  H  Y  R  K  L
181 AAGGTCCGTGTTGACATGGTTCGCATTCCGAGCATCAATGTTCATCATTATCGCAAACTG
 81  G  G  A  L  Q  A  K  A  D  R  P  H  E  R  N  G  F  T  V  L
241 GGCGGTGCGCTGCAGGCGAAAGCGGACCGTCCGCACGAGCGTAATGGCTTTACGGTGTTG
101  R  E  L  R  R  K  M  M  Y  M  A  D  V  T  E  H  D  T  L  L
301 CGCGAGCTGCGTCGTAAGATGATGTACATGGCGGACGTCACGGAACACGATACCCTGCTG
121  Y  K  N  L  V  P  M  V  A  T  V  K  V  L  E  E  T  S  V  M
361 TACAAAAACCTGGTCCCGATGGTTGCGACCGTGAAGGTGCTGGAAGAAACCAGCGTGATG
141  L  K  H  H  H  H  H  H  *
421 CTGAAACATCACCATCACCACCATTAA
```

Figure 15

H EBVpoly

```
  1  M  H  P  V  G  E  A  D  Y  F  E  Y  R  S  S  C  S  S  C  P
  1 ATGCATCCAGTTGGTGAAGCAGACTACTTTGAATACCGTTCCTCTTGCAGCTCGTGTCCG
 21  L  S  K  I  A  D  R  P  P  I  F  I  R  R  L  K  F  L  R  G
 61 CTGAGCAAGATTGCAGATCGTCCGCCGATCTTCATCCGTCGTTTGAAATTTCTGCGCGGT
 41  R  A  Y  G  L  R  G  L  C  T  L  V  A  M  L  A  D  E  E  C
121 CGCGCGTACGGCTTGCGTGGTCTGTGCACCCTGGTGGCCATGCTGGCGGACGAGGAGTGT
 61  D  S  E  L  E  I  K  R  Y  K  C  L  G  G  L  L  T  M  V  A
181 GATAGCGAGCTCGAAATCAAACGCTATAAGTGCCTGGGTGGCCTTCTGACGATGGTTGCT
 81  D  R  A  K  F  K  Q  L  L  R  A  T  I  G  T  A  M  Y  K  A
241 GACCGTGCGAAGTTTAAGCAACTGCTGCGCGCCACCATTGGTACGGCAATGTATAAAGCT
101  D  T  Y  G  P  V  F  M  C  L  K  L  P  E  P  L  P  Q  G  Q
301 GACACCTATGGCCCGGTTTTCATGTGTCTGAAGCTGCCGGAGCCGCTGCCGCAGGGTCAA
121  L  T  A  Y  K  I  E  D  P  P  F  N  S  L  A  D  V  S  F  I
361 CTGACCGCATACAAGATTGAGGACCCGCCGTTCAATAGCCTGGCGGACGTGAGCTTCATT
141  E  F  V  G  W  K  E  E  N  L  L  D  F  V  R  F  M  G  V  K
421 GAATTTGTCGGCTGGAAAGAAGAGAATTTGCTGGACTTCGTCCGCTTCATGGGCGTGAAA
161  Q  N  G  A  L  A  I  N  T  F  R  P  Y  L  F  W  L  A  A  I
481 CAGAACGGTGCTCTGGCAATCAACACGTTTCGTCCGTACCTGTTCTGGCTGGCGGCCATT
181  R  A  Y  S  S  W  M  Y  S  Y  A  D  R  V  R  A  Y  T  Y  S
541 CGTGCGTATAGCAGCTGGATGTACAGCTATGCCGATCGTGTCCGCGCGTACACCTACTCC
201  K  A  D  R  R  I  Y  D  L  I  E  L  R  V  E  I  T  P  Y  K
601 AAAGCGGATCGTCGTATCTACGATCTGATCGAGCTGCGTGTTGAAATTACCCCGTATAAA
221  P  T  W  A  D  H  H  H  H  H  H  *
661 CCTACTTGGGCGGATCACCATCATCACCACCACTAA
```

Figure 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2013/001216

A. CLASSIFICATION OF SUBJECT MATTER

C07K 19/00 (2006.01) A61K 39/12 (2006.01) A61P 31/22 (2006.01)

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)

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)

GenomeQuest search of SEQ ID NOS: 1-41.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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[X] Further documents are listed in the continuation of Box C [X] See patent family annex

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Date of the actual completion of the international search	Date of mailing of the international search report
18 December 2013	18 December 2013

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INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	**PCT/AU2013/001216**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/007556 A1 (THE COUNCIL OF THE QUEENSLAND INSTITUTE OF MEDICAL RESEARCH) 22 January 2004 Abstract; SEQ ID NOS: 1-2 and 94-97; page 20, line 25 – page 21, line 12; examples 3 and 4; page 14, line 21 – page 15, line 3; page 13, lines 1-16.	1-3, 9-10, 12-16, 18-22, 24-31
X	WO 2005/007689 A1 (ALPHAVAX, INC.) 27 January 2005 Abstract; SEQ ID NO 4; page 14, line 16 – page 15, line 11; examples 2-4; page 19, line 16 – page 20, line 3; claims 17-28.	1-6, 12-16, 18-22, 24-31
X	WO 2006/056027 A1 (THE COUNCIL OF THE QUEENSLAND INSTITUTE OF MEDICAL RESEARCH) 01 June 2006 Abstract; SEQ ID NOS: 1, 2, and 5; Figures 1, 11, and 12; page 16, line 22 – page 17, line 3; page 18, line 21 – page 20, line 22; page 20, line 23 – page 24, line 18; page 24, line 19 – page 25, line 8; claims 43-46.	1-7, 12-16, 18-22, 24-31
X	WO 2010/014567 A2 (MERK & CO., INC.) 04 February 2010 Abstract, SEQ ID NOS: 20-27; example 6, pages 51-64; page 21, line 10 – page 24, line 27; page 24, line 28 – page 28, line 5.	1-6, 12-16, 18-22, 24-31

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2013/001216**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
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		WO 2010014567 A2	04 Feb 2010

End of Annex

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