

Induction of innate immune signatures following polyepitope protein-glycoprotein B-TLR4&9 agonist immunization generates multifunctional CMV-specific cellular and humoral immunity

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Abbreviations: TLR, Toll-like receptors; CMV, cytomegalovirus, HLA, human leukocyte antigen; DC, dendritic cells; CMVpoly, CMV polyepitope; CMV-gB, CMV glycoprotein B

Recent studies have suggested that a successful subunit human cytomegalovirus (CMV) vaccine requires improved formulation to generate broad-based anti-viral immunity following immunization. Here we report the development of a non-live protein-based vaccine strategy for CMV based on a polyepitope protein and CMV glycoprotein B (gB) adjuvanted with TLR4 and/or TLR9 agonists. The polyepitope protein includes contiguous multiple MHC class I-restricted epitopes with an aim to induce CD8⁺ T cell immunity, while gB is an important target for CD4⁺ T cell immunity and neutralizing antibodies. Optimal immunogenicity of this bivalent non-live protein vaccine formulation was dependent upon the co-administration of both the TLR4 and TLR9 agonist, which was associated with the activation of innate immune signatures and the influx of different DC subsets including plasmacytoid DCs and migratory CD8-DEC205+CD103-CD326-langerin-negative dermal DCs into the draining lymph nodes. Furthermore these professional antigen presenting cells also expressed IL-6, IL-12p70, TNF α , and IFN α which play a crucial role in the activation of adaptive immunity. In summary, this study provides a novel platform technology in which broad-based anti-CMV immune responses upon vaccination can be maximized by co-delivery of viral antigens and TLR4 and 9 agonists which induce activation of innate immune signatures and promote potent antigen acquisition and cross-presentation by multiple DC subsets.

Introduction

The development and widespread use of vaccines represents one of the most significant achievements of modern medicine and they have been instrumental in controlling and even eliminating life-threatening diseases including smallpox, polio, diphtheria, and measles. However, despite these successes there are a number of complex disorders (cancers, HIV, malaria, tuberculosis and other emerging infectious diseases) for which effective or preventive vaccines have not been developed. Over the past decade, several reports have specified new emerging mechanistic insights into how the innate immune system triggers protective immune responses and regulates the magnitude, quality, and persistence of vaccine-induced immunity.¹⁻⁵ The most significant of these new insights include the identification of pathogen recognition receptors (PRRs),² the identification and transcriptional regulation of the various dendritic cells (DCs) subsets,⁶ and the role of distinct subsets of helper T cells, in protecting against

different pathogens.^{7,8} Therefore, a rational approach for directing vaccine responses toward a number of effector cell subsets of the immune system will likely improve vaccine efficacy and these mechanistic insights will potentially provide tools for designing better vaccines and improving the protective efficacy of existing vaccines.

Human Cytomegalovirus (CMV) is a widely prevalent β herpesvirus. In healthy individuals after primary infection CMV establishes a latent state with periodical reactivation and shedding from mucosal surfaces. Although primary infection is generally asymptomatic, CMV can cause significant morbidity and mortality in immunosuppressed individuals through reactivation of latent virus or reinfection with a different isolate. Congenital infection, primarily through intrauterine transmission, is a leading cause of birth defects in children.⁹⁻¹² Based on the life-time cost to the health care system and its impact on human suffering, development of a vaccine to prevent congenital CMV infection has been assigned the highest priority by the Institute of

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Results

Rational design of the CMV polyepitope constructs, protein expression, purification, and in vitro immunogenicity studies

The CMV polyepitope was designed to encode 13 minimal CD8⁺ T cell epitopes without (CMVpoly) and with (CMVpoly-L) spacer sequences (Fig. 1A and B). The spacer sequences were designed based upon previous observations demonstrating the enhanced liberation of T cell peptide epitopes following the inclusion of a proteasome liberation amino acid sequence and a TAP recognition motif.¹⁸ The CMV polyepitope constructs were transformed into *E. coli*, protein expression conditions were optimized, and polyepitope proteins were purified using Ni-NTA chromatography. Results obtained from these experiments showed that both the CMVpoly and CMVpoly-L could be successfully expressed and purified to homogeneity using a bacterial expression system (Fig. 2A).

To investigate the processing and presentation of the CMVpoly and CMVpoly-L proteins, we incubated human lymphoblastoid cell lines (LCLs) overnight with CMVpoly or CMVpoly-L, and then assessed the activation of a panel of CMV-specific T cells using intracellular IFN- γ analysis.

Representative data presented in Figure 2B shows that HLA A2-restricted pp65 epitope, NLVPMVATV (referred to as NLV), HLA A1-restricted pp50 epitope, VTEHDLLY (referred to as VTE), HLA B7-restricted pp65 epitopes RIPHERNGFTVL (referred to as RPH), and TPRVTGGGAM (referred to as TPR) from CMVpoly-L were more efficiently processed and presented to CMV-specific T cells compared with LCLs pulsed with CMVpoly. To extend this analysis, we compared the processing and presentation of CMVpoly-L protein with full-length CMV proteins, including pp65 and IE-1 proteins. Data presented in Figure 2C shows that the HLA A2-restricted epitopes NLV and VLEETSVML (referred to as VLE) and HLA B7-restricted RPH epitope from CMVpoly-L protein were more efficiently processed and presented to CMV-specific T cells compared with the epitopes presented from the full-length pp65 or IE-1 proteins.

To further evaluate the immunogenicity of the CMVpoly-L protein, PBMC from 10 CMV-seropositive individuals, HLA matched for the epitopes expressed in CMVpoly-L, were incubated with the CMVpoly-L protein then cultured for 10 d in the presence of IL-2. The expansion of epitope specific T cells was then assessed by intracellular cytokine assays (ICS) assay. Representative data from one of these ICS assays is presented Figure 3A. The CMVpoly-L protein induced expansion of CMV specific CD8⁺ T cell specific in all 10 individuals and these expansions ranged from 8–8900 fold (Fig. 3B). In the majority

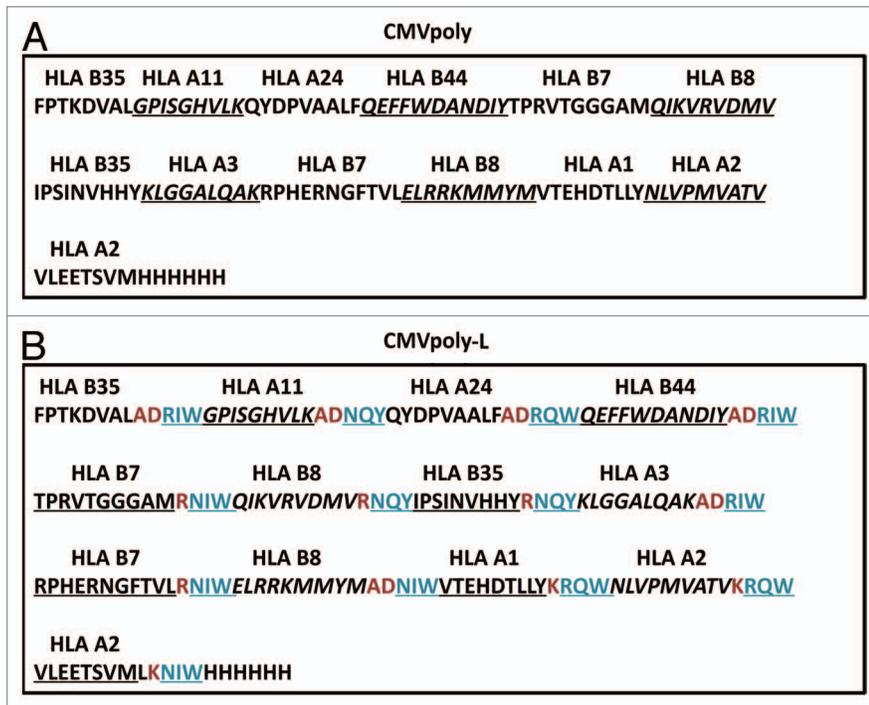


Figure 1. Schematic design of the CMV polyepitope protein construct with and without linkers. **A** shows the design of CMV polyepitope protein without linkers (referred to as CMVpoly), while **B** shows the design of polyepitope protein with linkers (referred to as CMVpoly-L). Each of the alternate CD8⁺ T cell epitope sequences are italicized and underlined. HLA restriction of the every epitope is specified above epitope sequence. For CMVpoly-L each epitope sequence is separated by amino acid residue(s) which are targets for proteasomal degradation (shown in red) and a peptide transporter recognition motif (shown in blue).

Medicine of the National Academy of Sciences (US) and US National Vaccine Program Office. (i.e., most favorable impact—saves both money and quality-adjusted life years [QALYs]).¹³

Recent insights in understanding the immunology, pathology, and molecular biology of CMV suggests that protection against CMV-related disease is mediated by both humoral and cellular immunity, thus, an ideal vaccine against CMV needs to induce both humoral and cellular responses.^{14,15} We have previously shown that an adenoviral vector encoding polyepitope combined with CMV encoded glycoprotein B (CMV-gB) protein to target the humoral and cellular mediated immune responses is one of the most efficient strategies for inducing CMV-gB-specific antibodies and CD4⁺ T cell responses and a broad spectrum of CD8⁺ T cell responses.¹⁶ However, the issues of pre-existing immunity and safety of the use of viral vectors have impeded implementation of this vaccine in a clinical setting.¹⁷ To overcome these major roadblocks, we have developed a novel platform technology to express multiple CMV CD8⁺ T cell epitopes as a polyepitope protein using a prokaryotic expression system and combined this with recombinant CMV glycoprotein B (gB) to generate a novel bivalent non-live protein-based vaccine formulation. In this study we have demonstrated that this bivalent vaccine formulation based on polyepitope protein and gB adjuvanted with TLR agonists can be successfully used to induce CMV-specific cellular and humoral immune responses.

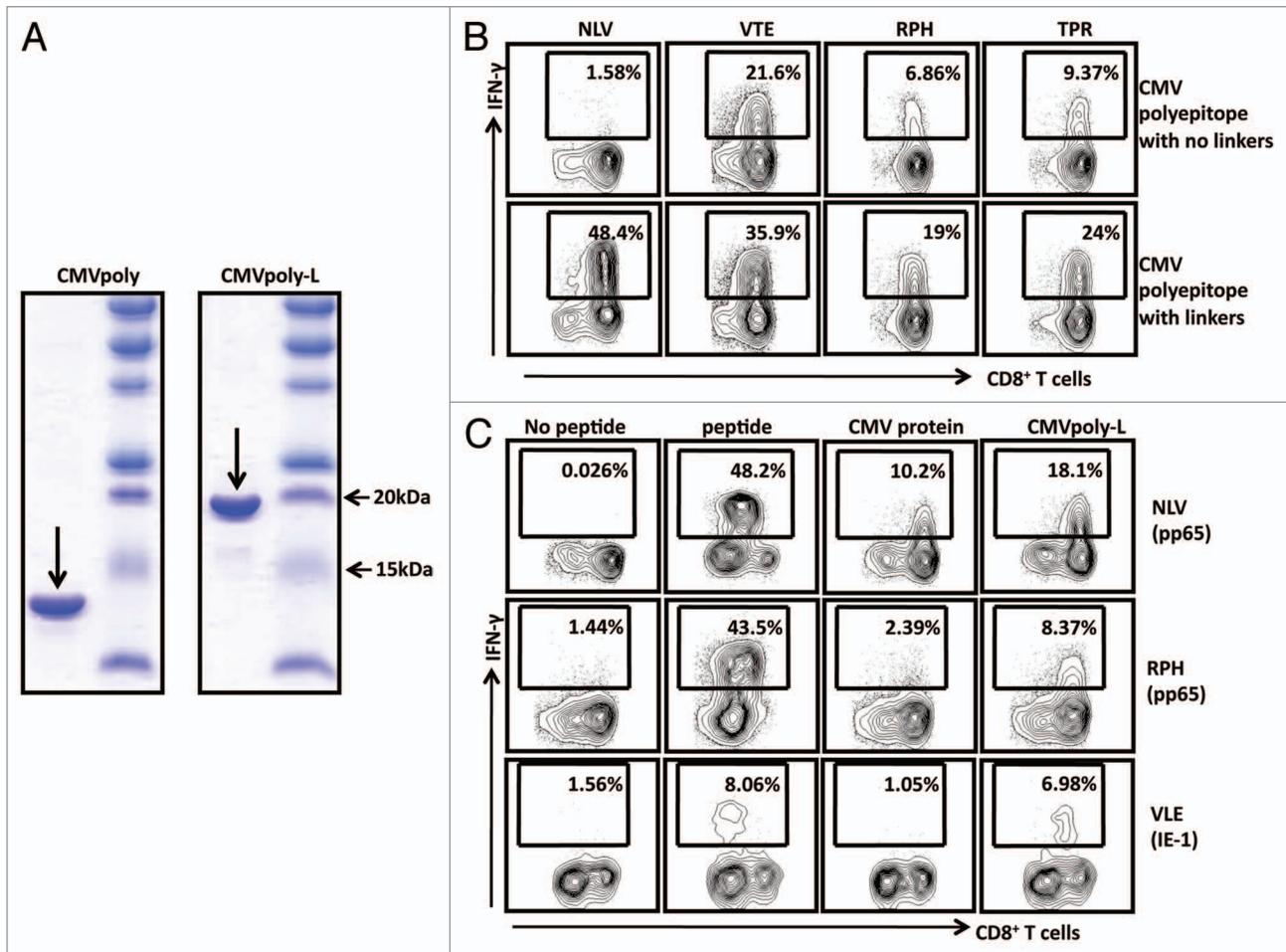


Figure 2. CMV polyepitope protein purification and in vitro assessment of processing and presentation by human cells. The DNA sequence encoding the CMV polyepitope proteins was cloned into an IPTG inducible plasmid, pJexpress 404, and transformed into *E. coli* for protein expression. Polyepitope protein was purified using Ni-NTA affinity chromatography. **A** shows SDS-PAGE analysis of purified CMVpoly and CMVpoly-L proteins. Predicted size for the CMVpoly and CMVpoly-L was 14.7Kd and 19.4Kd respectively. **B** shows in vitro cross-presentation of CMV polyepitope protein by human cells. EBV transformed human lymphoblastoid cell lines (LCLs) were pulsed with CMVpoly or CMVpoly-L protein (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 B7-restricted RPH (pp65), and HLA B7-restricted TPR (pp65) epitopes. The FACS plots show IFN-γ expression by the CMV-specific CD8⁺ T cells following co-culture with CMVpoly or CMVpoly-L proteins pulsed LCLs. **C** shows comparative activation of CMV-specific T cells by the synthetic peptide epitopes, full-length recombinant CMV proteins (pp65 or IE-1) and CMVpoly-L protein. EBV-transformed LCLs were sensitized with synthetic peptide epitopes, full-length pp65 or IE-1 proteins or CMVpoly-L protein as indicated above and then exposed to CMV-specific CD8⁺ T cells. Following incubation, these CD8⁺ T cells were assessed for IFN-γ expression by ICS assays. The FACS plots show IFN-γ expression by CMV-specific CD8⁺ T cells following co-culture with antigen loaded LCLs. This data are representative of one of the three independent sets of experiments.

of the individuals expansion of T cells directed toward multiple epitopes was observed. Taken together, these data demonstrate the enhanced capacity of the CMVpoly-L protein to deliver CMV-restricted T cell epitopes for presentation to human CD8⁺ T cells.

CMV vaccine formulation with TLR4 and TLR9 agonists promote recruitment, activation of DC subsets and innate immune signatures in draining lymph nodes

The efficient delivery of antigen in the context of the correct innate inflammatory signals is a critical component of any vaccine platform targeting the induction of adaptive immunity. Indeed a number of recent studies have successfully integrated systems biology approaches into vaccinology to identify innate

immune signatures that are associated with vaccine efficacy.¹⁹⁻²¹ To identify the most appropriate combination of adjuvants for the CMV vaccine, we first assessed the impact of the synergistic activity of TLR4 and TLR9 adjuvanted CMV vaccine on the recruitment of professional antigen presenting cells into draining lymph nodes (DLN) which may play a crucial role in the induction of adaptive immunity. Using a panel of antibodies specific for the markers CD11c, CD8, B220, DEC205, CD103, and CD326 we identified a total of six distinct populations of CD11c⁺ DCs based on the differential expression of these markers similarly described in a previous study.²² The gating strategy for each of these DC subpopulations is shown in Figure 4A. These populations included the resident CD11c⁺CD8⁺ (CD8+DCs,

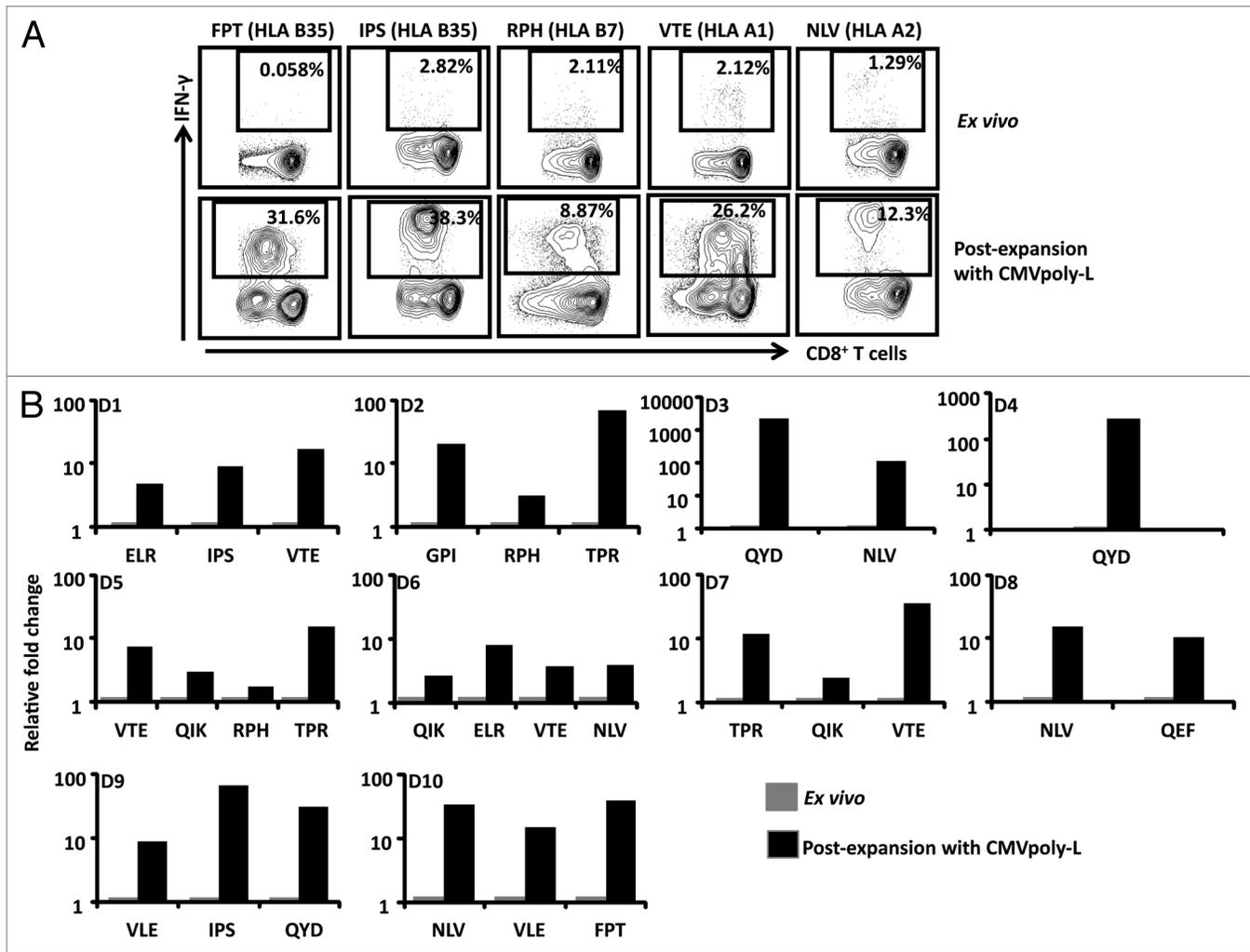


Figure 3. In vitro expansions of CMV-specific T cells from healthy virus carriers following stimulation with the CMVpoly-L protein. PBMC from ten different healthy CMV-seropositive individuals were stimulated with recombinant CMVpoly-L protein and cultured for 10 d in the presence of recombinant IL-2. The percentage of expanded peptide specific CD8⁺ T cells producing IFN- γ was determined using an ICS assay. **A** shows the representative FACS plots illustrating IFN- γ expression by in vitro expanded CMV-specific CD8⁺ T cells following stimulation with CMVpoly-L protein. **B** shows the fold change in in vitro expanded CMV-specific CD8⁺ T cells relative to ex vivo responses from ten different CMV-seropositive donors (D1 to D10). The fold expansion of T cells for each donor was calculated using the T cell numbers before and after stimulation with CMVpoly-L protein.

subset 1), CD11c⁺B220⁺(pDCs, subset 2), CD11c⁺CD8⁻DEC205⁺CD103⁻CD326⁻, (Langerin negative dermal DCs, subset 3), CD11c⁺CD8⁻DEC205⁺CD103⁻CD326⁺ (Epidermal Langerhans cells, subset 4), CD11c⁺CD8⁻DEC205⁺CD103⁺ (Langerin positive dermal DC, subset 5), and CD11c⁺CD8⁻DEC205⁻ (resident blood derived DCs, subset 6). Data presented in **Figure 4B** shows that while we saw little impact on the number of epidermal langerhans cells and Langerin⁺ dermal DCs in the draining lymph nodes, immunization with the TLR4 agonist MPL lead to an increase in the frequency of Langerin⁻ dermal DCs, pDCs, and resident blood derived DC, whereas immunization with the TLR9 agonist CpG produced a more modest increase in the frequency of Langerin⁻ dermal DCs and a reduction in the frequency of pDCs and CD8⁺ DCs. Interestingly, immunization with a combination of the TLR4 and TLR9 agonists induced a similar increase in the frequency of Langerin⁻ dermal

DCs and pDCs to that seen with MPL alone. These observations suggested that the synergistic benefit provided by the TLR4 agonist MPL when delivered in combination with CpG, is through the enhanced trafficking of dendritic cells, particularly the skin derived dermal DC, into the DLN. To further address the impact the TLR agonists have upon antigen processing and presentation by professional antigen presenting cells, CD11c⁺ DCs were loaded with the CMVpoly-L protein, activated with the TLR agonists, then assessed for their capacity to present peptide to a T cell hybridoma specific for the HLA A2 restricted VLE epitope. Pre-treatment with the TLR9 agonist CpG alone or in combination with MPL, enhanced the cross-presentation of CMVpoly-L however, MPL failed to enhance the CMVpoly-L cross-presentation (**Fig. 4C**).

We further delineated the impact of the TLR4 and/or TLR9 agonist on the molecular innate immune signatures in vivo using

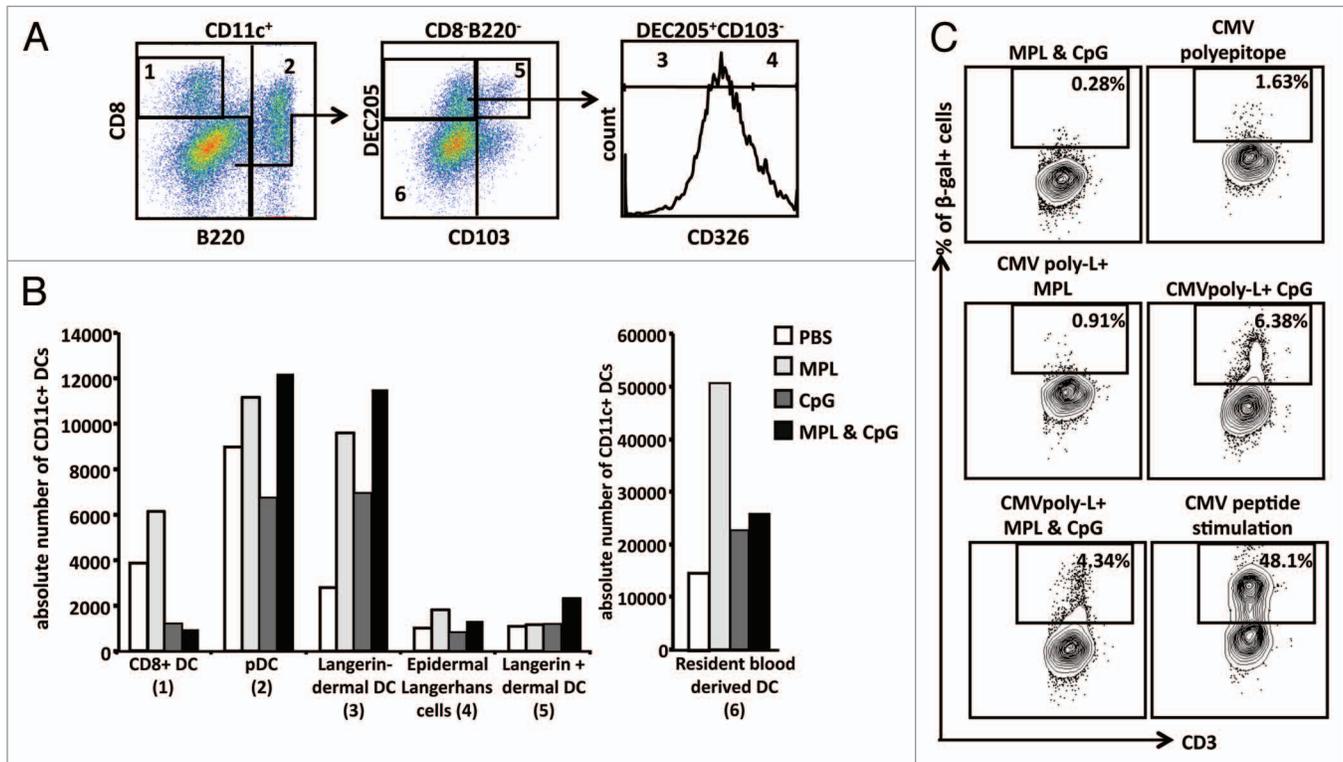


Figure 4. Assessment of recruitment of distinct DC subpopulations in the DLN of HHD-I mice following vaccination with different TLR agonists. HHD-I mice were vaccinated with PBS, TLR4, TLR9, or TLR4 and TLR9. Following 48 h of vaccination CD11c⁺ DCs were enriched from inguinal lymph nodes. **A** shows representative FACS plots of different subsets of CD11c⁺ DCs. These cells were divided into subset 1 (CD8⁺ DCs), subset 2 (pDCs) based on the expression of CD8 and B220, subset 3 (Langerin negative dermal DCs) based on the expression of CD8⁺DEC205⁺CD103⁺CD326⁺, subset 4 (Epidermal Langerhans cells) based on the expression of CD8⁺DEC205⁺CD103⁺CD326⁺, subset 5 (Langerin positive dermal DC) based on the expression of CD8⁺DEC205⁺CD103⁺ and subset 6 (resident blood derived DCs) based on the expression of CD8⁺DEC205⁺. **B** shows the absolute number of different DC populations recruited in the draining lymph nodes of HHD-I mice vaccinated with different TLR ligands. Lymph nodes from 5 mice in each group were pooled for these analyses. **C** shows representative FACS plots of cross-presentation of CMVpoly-L protein by CD11c⁺ in the presence of different TLR agonists. CD11c⁺ DCs were pulsed with 25 μg of CMVpoly-L for two hours and then washed and resuspended in DC growth medium containing no adjuvant, TLR4, TLR9, or TLR4 and TLR9 incubated over night. CMVpoly-L pulsed CD11c⁺ DCs were mixed with β-gal expressing HLA A2-restricted IE-1 VLE epitope-specific murine T cell hybridoma cells and incubated for 6 h and then β-gal activity was determined using FDG substrate. Cells stimulated with adjuvants alone used as negative control and cells pulsed with VLE peptide used as a positive control. Representative data from one of the two independent experiments is presented here.

a customized gene expression array published by Kwisa and colleagues.⁶ Cells from DLN of HHD-I mice were harvested 24 h and 48 h after injection with different TLR ligands and assessed for the expression of a panel of genes associated with innate immune activation. Data presented as a gene-expression heat map in **Figure 5A** showed that 60 out of 92 genes were at least 2-fold up- or downregulated from baseline (PBS control) at 24 h post injection in TLR4 agonist alone, TLR9 agonist alone or TLR4 and TLR9 agonist groups (n = 4). While the mice immunised with TLR4 agonist alone stimulated a weak transcriptional signature, the TLR9 agonist alone or in combination with the TLR4 agonist significantly upregulated transcription of several genes. Consistent with the data presented in **Figure 4**, significant upregulation of expression of genes encoding inflammatory cytokines including *IL12B*, *IL6*, and *IL1A* was observed (**Fig. 5B**). In addition, other signature genes including the type I IFN-stimulated genes (ISGs) (*ISG-15*, *Iffit1B*); chemokines involved in T cell recruitment (*Cxcl-10*, *Cxcl-11*, *Ccl-13*) and interferon related genes (IRF) (*IRF7*) were also upregulated (**Fig. 5B**). These genes

are known to promote T helper 1 cell development and the induction of a CD8⁺ T cell response. Collectively, these data indicate that TLR9 alone or a TLR4 and TLR9 combination has the ability to induce robust and sustained activation of professional antigen presenting cells and expression of genes encoding proteins involved in the initiation of inflammatory and antiviral responses in the DLN when compared with the TLR4 agonist MPL alone.

Finally we assessed the functional impact of different TLR agonist combinations have upon the activation of murine DCs. Murine CD11c⁺ DCs were cultured in vitro in the presence of the TLR agonists, MPL (TLR4) and/or CpG (TLR9) and the cell culture supernatants were assessed for the expression of the pro-inflammatory cytokines IL-6, IL-12p70, TNF, and IFN-α using ELISA. In addition, the intracellular expression of IL-12p70 was also assessed using an ICS assay. Data presented in **Figure 6A and B** shows that the TLR9 agonist alone or in combination with TLR4 agonist induced optimal expression of IL-12, IL-6, IFN-α, and TNF. More importantly, the combination of the TLR4 and TLR9 agonists showed a synergistic effect on IL-12

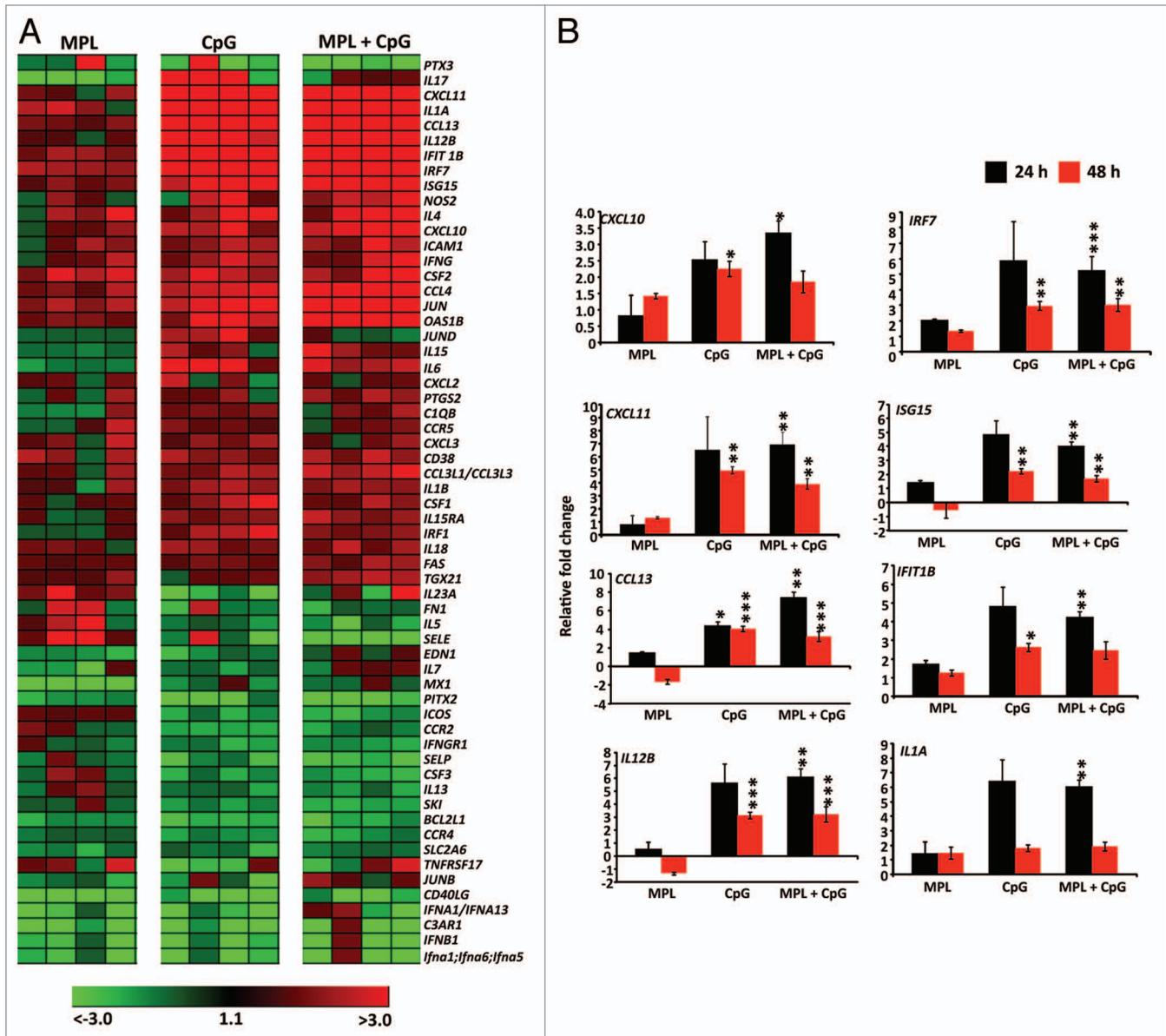


Figure 5. Pro-inflammatory innate gene transcription signature following administration of different TLR agonists. HHD-I mice ($n = 4$ in each group) were immunized with PBS, TLR4, TLR9, or TLR4 and TLR9 agonists. RNA was isolated after 24 h and 48 h from draining lymph nodes and then subjected to low-density array real-time PCR for a panel of 92 genes involved in innate immune responses. All genes were first normalized to the average Ct values of expression of the house keeping genes for *18S*, *Actb* and *Gusb*. **A** shows heat map of genes upregulated and downregulated at least 2-fold relative to the PBS group following 24 h of immunization. Each column represents the experimental group ($n = 4$) and row represent the fold change of individual genes. **B** shows summary of significantly upregulated and downregulated genes relative to PBS group at the 24 and 48 h time point is shown in the bar graphs. Bar graphs represent the mean \pm SEM. P values were calculated by student's *t* test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (determined by the Student *t* test).

production by plasmacytoid DCs compared with the cells treated with TLR4 or TLR9 alone (Fig. 6A). In summary, these data establish that CMVpoly-L protein in combination with TLR4 and TLR9 agonists induce a unique set of innate immune signatures which influence DC activation and migration into the DLN following immunization and enhance cross-presentation of antigen by CD11c⁺ DCs.

CMV vaccine formulated with TLR4 and 9 agonists is required for multifunctional Th1 CD4⁺ T cell and CD8⁺ T cell immunity

It is now firmly established that a successful subunit CMV vaccine requires improved formulation to generate broad-based anti-viral immunity including both cellular (CD4⁺ and CD8⁺ T cells) and humoral immunity. Data presented above indicated

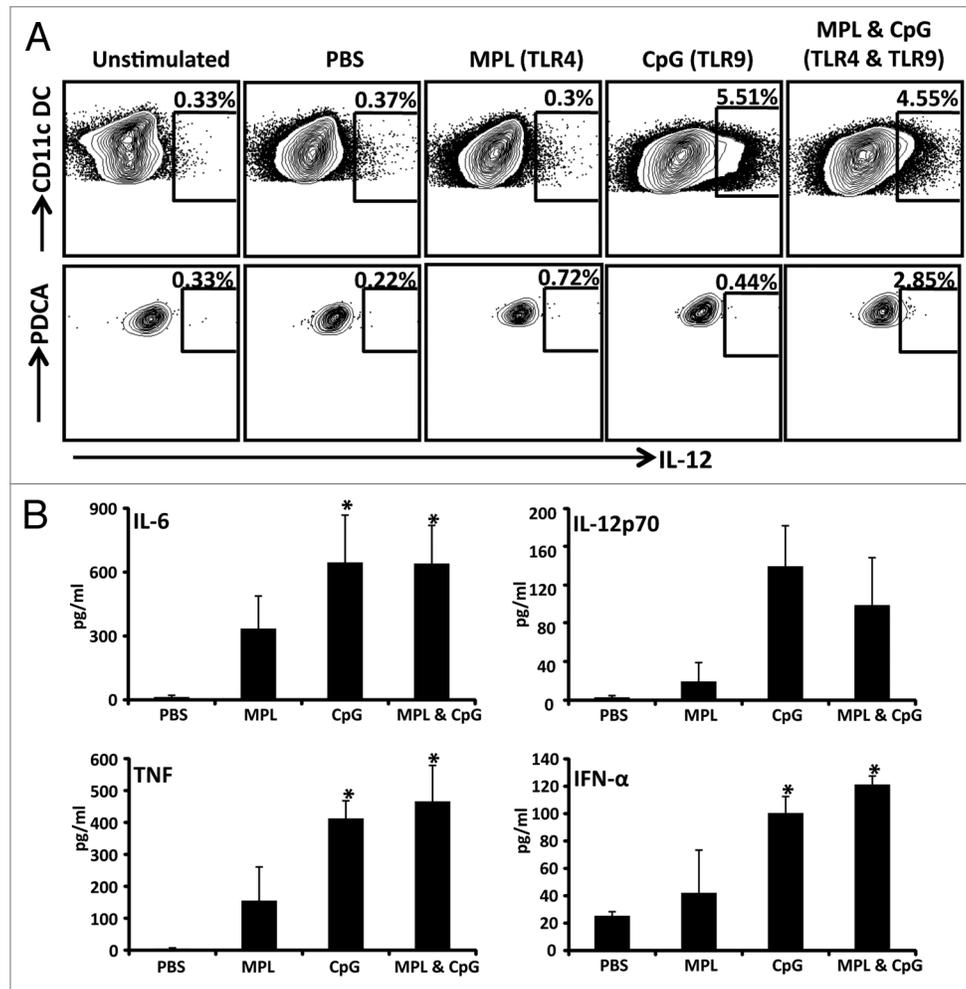


Figure 6. Assessment of proinflammatory cytokine production following DC stimulation with various adjuvant combinations. CD11c⁺ DCs were enriched using CD11c Microbeads and stimulated with PBS, TLR4 agonist alone, TLR9 agonist alone, or the TLR4 and TLR9 agonists for 20 h in a 48 well plate. **A** shows FACS plots of CD11c⁺ DCs and pDCs producing IL-12p70 following stimulation with indicated adjuvants. **B** shows the levels of pro-inflammatory cytokines IL-6, IL-12p70, TNF-α and IFN-α by CD11c⁺ DCs following stimulation with PBS, TLR4 agonist alone, TLR9 agonist alone, or the TLR4 and TLR9 agonists. These cytokines were assessed in culture supernatants using standard ELISA assays. The data represent the mean ± SEM from two independent experiments. Error bars represent the mean ± SEM *P < 0.05 (determined by the Student t test).

that a vaccine formulation adjuvanted with TLR agonists may provide an appropriate inflammatory environment to induce an effective immune response in vivo. To assess this hypothesis, we formulated a bivalent CMV vaccine which included CMVpoly-L protein (to induce CD8⁺ T cell responses) and the CMV-gB protein (to induce CD4⁺ T cell responses and anti-viral neutralizing antibody responses) in combination with the TLR4 and/or TLR9 agonists. In the first set of experiments, we assessed the ability of this vaccine formulation to induce CMV-specific cellular immunity. HHD-I mice were immunized with this vaccine formulation and then boosted with the same formulation on day 21 and sacrificed on day 50. To analyze the establishment of T cell memory, splenocytes were assessed for the presence of CMV-gB specific CD4⁺ T cells, and CMVpoly-L specific (HLA A2-restricted epitopes NLV and VLE) CD8⁺ T cells using an intracellular IFN-γ assay. CMV vaccine formulation (CMVpoly-L and CMV-gB) adjuvanted with a combination of

a TLR4 and TLR9 agonist was required to induce an optimal T cell response (Fig. 7A and B). While the induction of a CMV-gB-specific CD4⁺ T cell response could be detected ex vivo following vaccination in the presence of the TLR4 or TLR9 agonists, these responses were significantly enhanced when CMV vaccine was adjuvanted with both TLR agonists (Fig. 7B). In contrast, CMV-specific CD8⁺ T cell responses were detected in mice immunized with CMV vaccine adjuvanted with CpG alone or MPL and CpG. CD8⁺ T cell responses were significantly lower in mice immunized with CMV vaccine adjuvanted with MPL alone. To confirm these observations, splenocytes from these immunized mice were stimulated with the HLA A2-restricted T cell synthetic peptides epitopes, NLV and VLE for 7 d, and then assessed for the frequency of IFN-γ producing CD8⁺ T cells. There was a dramatic expansion of CMV-specific CD8⁺ T cells from mice immunized with CMV vaccine adjuvanted with CpG alone or CpG and MPL (Fig. 7A and B).

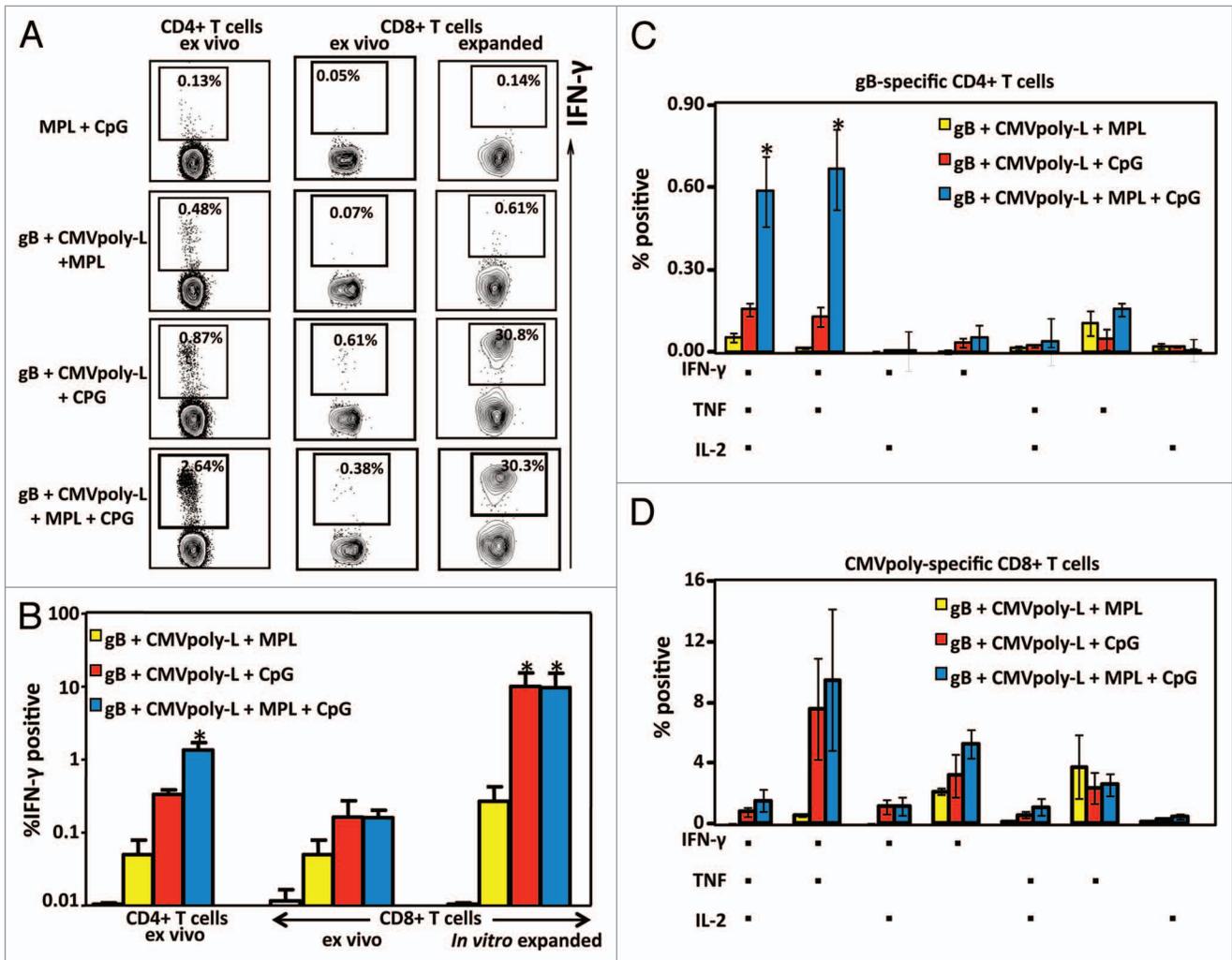


Figure 7. Evaluation of virus-specific CD4⁺ and CD8⁺ T cell responses following immunizations with the CMV vaccine adjuvanted with TLR agonists. HHD-I mice (n = 5 in each group) were immunized with CMV vaccine formulation on day 0 and then boosted with same formulation on day 21. On Day 50, splenocytes from immunized mice were stimulated in vitro with CMV-gB protein or HLA A2-restricted NLV (pp65) and VLE (IE-1) peptide epitopes in the presence of brefeldin A, then IFN-γ production by CD4⁺ and CD8⁺ T cells was measured using an ICS assay. **A** shows representative FACS plots of the frequency of IFN-γ producing CMV-gB specific CD4⁺ T cells and CMV-specific CD8⁺ T cells (ex vivo and in vitro expanded). **B** shows comprehensive analysis of CMV-specific IFN-γ producing CD4⁺ and CD8⁺ T cell responses. **C** shows multifunctional potentiality of CMV-gB specific CD4⁺ T cells and CMV-specific CD8⁺ T cells. On day 50, splenocytes from immunized mice were in vitro stimulated with CMV-gB protein in the presence of brefeldin A and production of IFN-γ, TNF-α, or IL-2 was determined using multiparameter ICS. Panel D shows multifunctional potentiality of CMV-specific CD8⁺ T cells. On day 50, splenocytes from immunised mice were in vitro stimulated with CMV CD8⁺ T cell HLA A2-restricted peptide epitopes (NLV and VLE) for 10 d in the presence of IL-2 and then assessed to determine the induction IFN-γ, TNF-α, or IL-2 cytokines using multiparameter ICS. Following ICS data was analyzed using FlowJo software and percentage of antigen-specific CD4⁺ or CD8⁺ T cell inducing three cytokine, two cytokines, or one cytokine were plotted in the bar graph. Error bars represent the mean ± SEM *P < 0.05 (determined by the Student t test).

There is substantial evidence that the protective efficacy of T cell-based vaccines correlates with the frequencies of multifunctional effectors.²³⁻²⁵ To investigate the functional quality of the memory T cell response, we assessed the pattern of IL-2, TNF, and IFN-γ production using multiparametric flow cytometry either ex vivo for CMV-gB-specific CD4⁺ T cells or following in vitro expansion of CMVpoly-L specific CD8⁺ T cells (Fig. 7C and D). Both CMV-gB specific CD4⁺ T cells and CMVpoly-L specific CD8⁺ T cells displayed greater polyfunctionality in mice immunized with CMV vaccine adjuvanted with MPL and CpG or CpG alone. However, frequencies of gB-specific CD4⁺ T cells expressing

all three cytokines were significantly higher following vaccination with MPL and CpG compared with MPL alone and CpG alone. While a similar proportion of CMV-gB specific CD4⁺ T cells were capable of expressing all three cytokines, interestingly, a large proportion of CD4⁺ T cells from HHD-I mice immunized with CMV vaccine adjuvanted with MPL alone predominantly expressed TNF (Fig. 7C). Similarly a large proportion of CMVpoly-L specific CD8⁺ T cells from mice immunized with CMV vaccine formulation adjuvanted with MPL expressed only TNF (Fig. 7D). In contrast, vaccination with a vaccine formulation based on CpG or MPL and CpG predominantly generated CD8⁺ T cells which

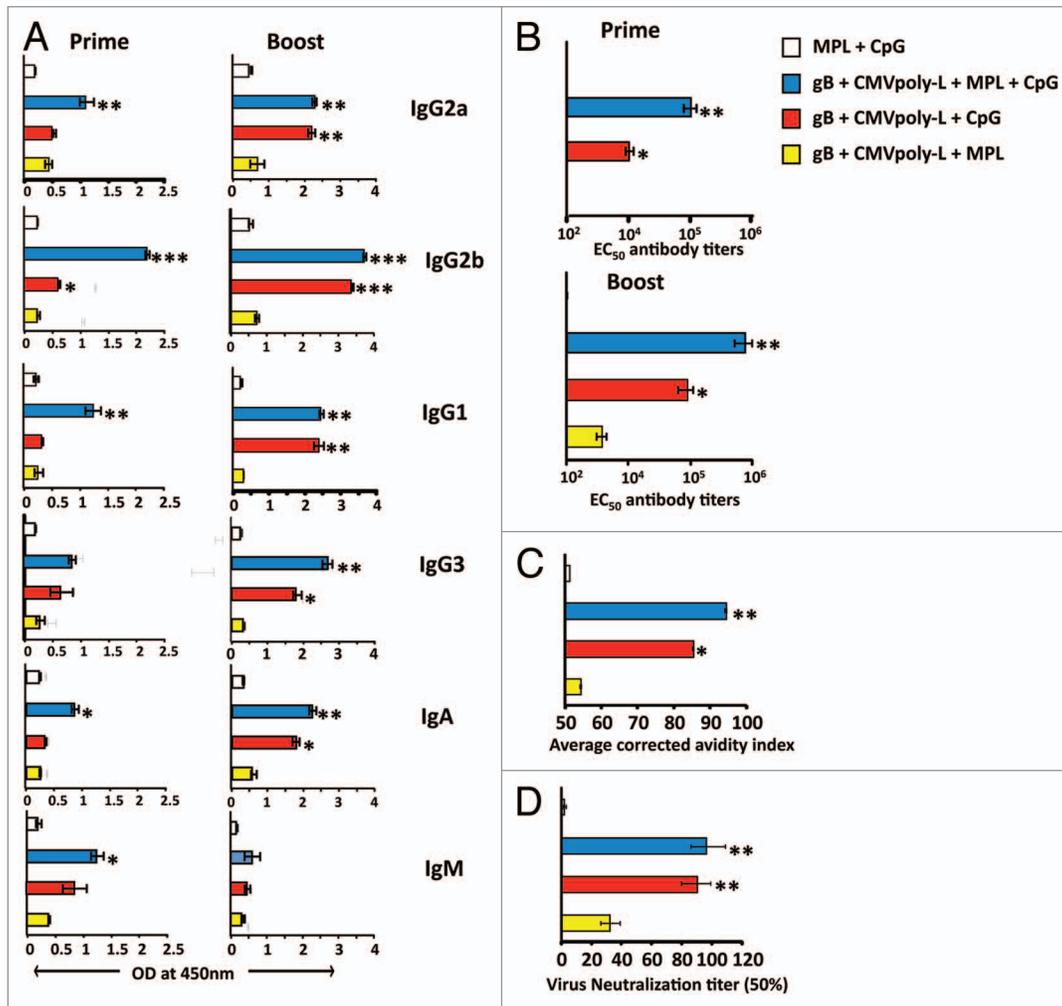


Figure 8. Assessment of CMV-specific humoral responses following vaccination with CMV vaccine adjuvanted with TLR agonists. To study the synergistic or antagonistic effect of TLR4 and TLR9, four groups (n = 5/group) of HHD-I mice were immunized subcutaneously with CMV-gB and CMVpoly-L proteins adjuvanted with TLR4 alone, TLR9 alone, or TLR4 and TLR9. On day 21 these mice were tail bled and a booster dose was given. Mice were sacrificed on day 50 to assess CMV-gB specific humoral immune responses. **A** shows gB-specific isotype (IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM) antibody responses in pooled serum samples from treatment groups assayed in triplicates on days 21 and 50. The data represent the mean \pm SEM *** P < 0.001, ** P < 0.01, and * P < 0.05 (calculated using student's t test). **B** shows the CMV-gB specific 50% effective concentration (EC_{50}) antibody titers determined by ELISA on day 21 in pooled sera and on day 50 in individual mouse serum. Error bars represent the mean \pm SEM ** P < 0.01. **C** shows avidity maturation of CMV-gB specific antibody responses. Pooled serum samples from immunized mice at day50 were tested for their CMV-gB specific binding avidity using urea dissociation avidity assay. Bar graphs represent the average corrected avidity index. * P < 0.05, *** P < 0.01. **D** shows 50% CMV-specific neutralizing antibody titers induced following immunization. On day 50, sera from individual groups were pooled, serially diluted, and pre-incubated with HCMV AD169 strain. MRC-5 cells were infected with serum-treated virus and virus infectivity was determined using an IE-1/IE-2 micro-neutralization assay. Neutralizing assay was performed in triplicates. ** P < 0.01.

were $IFN-\gamma^+TNF^+$ or $IFN-\gamma^+$ (Fig. 7D). Taken together these analyses demonstrated that a CMV vaccine formulation based on CMVpoly-L and CMV-gB proteins adjuvanted with both TLR4 and TLR9 agonist was most effective in inducing CMV-specific $CD4^+$ and $CD8^+$ T cells with a multifunctional capability.

Induction of anti-viral neutralizing antibody responses are synergistically enhanced by TLR4 and 9 agonists

In the next set of experiments we assessed quantitative and qualitative analysis of CMV-specific humoral immune response in animals immunized with bivalent CMV vaccine adjuvanted with different TLR agonists. The combination of TLR4 and TLR9 agonist induced high titer CMV-gB-specific antibody

response which included multiple isotypes, including the IgG2b, IgG3, IgG2a (Th1 like Ig isotypes), IgG1 (Th2 like Ig isotype), IgA, and IgM (Fig. 8A and B). The CMV vaccine formulated with the TLR4 and TLR9 agonist induced significantly higher titers of CMV-gB specific antibody responses following the primary immunization which were sustained at high levels until at least day 50 post-immunization, when compared with the vaccine formulated with CpG or MPL alone (Fig. 8B). To assess the quality of the CMV-gB specific antibody response generated following vaccination in the different adjuvant combinations, we assessed the functional avidity of CMV-gB specific antibody and the level of CMV-specific neutralizing antibody using a

micro-neutralization assay. Antibodies generated in mice vaccinated with the TLR4 and TLR9 agonists displayed significantly higher functional avidity compared with mice immunized with MPL alone, and to a lesser extent to mice immunized with CpG (Fig. 8C). Similarly, while antibodies from mice immunized with MPL alone failed to display a strong neutralizing response, the levels of anti-viral neutralizing responses were much greater in mice immunized with CMV vaccine adjuvanted with CpG, or MPL and CpG (Fig. 8D). These observations provide further evidence that a CMV vaccine formulation based on CMV polyepitope and CMV-gB proteins adjuvanted with TLR4 and TLR9 agonist provides the optimal strategy to induce both cellular and humoral immune responses.

Discussion

Following extensive efforts over the past 30 y, recombinant subunit CMV vaccine formulations (gB/MF59 and DNA vaccine encoding pp65 and gB) have emerged as the most promising candidates and have successfully concluded advanced clinical trials.²⁶⁻²⁸ In Phase II clinical trials gB/MF59 vaccine showed 50% efficacy in young women and in transplant patients these vaccine formulations reduced viral reactivation.²⁹ However, these vaccine formulations were unable to eliminate the need for antiviral therapy in transplant patients or reduce the incidence of CMV disease.^{30,31} Therefore, a vaccine with higher efficacy to prevent CMV-associated clinical disease would be highly advantageous. In immunocompetent individuals CMV infection is maintained under strict control by the immune system by a combination of humoral and cellular immune responses. Thus, the major criteria for the design of an effective CMV vaccine should be the induction of virus-specific antibodies as well as CD4⁺ and CD8⁺ T cell responses.^{14,32,33}

In our previous studies we investigated a vaccine formulation combining CMV-gB with a TLR9 agonist and immune stimulating complexes (AbISCO®100).³⁴ Although this CMV-gB vaccine was able to induce antibody and cellular immune responses, a single antigen is unlikely to be broadly effective due to the complexity of CMV immunity. In addition, emerging evidence suggests that CMV-specific CD8⁺ T cell responses in healthy CMV-seropositive individuals are directed toward multiple CMV antigens, predominantly pp65 and IE1, but also other structural, early/late antigens and immunomodulators.³⁵⁻³⁹ Therefore, we employed a novel platform technology that allows the activation of CMV-specific CD8⁺ T cell responses directed toward multiple epitopes restricted through a range of HLA class I molecules. Using a prokaryotic expression system, multiple minimal HLA class I-restricted CD8⁺ T cell epitopes were expressed as a polyepitope protein. Interestingly, we found that human antigen-specific CD8⁺ T cells were more efficiently activated by the polyepitope protein which included proteasomal/TAP targeting flanking sequences (CMVpoly-L). These observations were in contrast to our previously published data which showed that flanking sequences are not required when a polyepitope is delivered endogenously in the context of a viral vector.^{40,41}

Furthermore, we also showed that the polyepitope protein-based antigen delivery was more efficient in activating human CD8⁺ T cells when compared with the full-length CMV proteins.

The ability of a vaccine to skew the immunological response toward a specific type is highly important for the induction of pathogen-specific immune responses.²² Emerging evidence suggests that activation of resting DCs that migrate to draining lymph nodes, produce pro-inflammatory cytokines and present antigens to T cells are critical for the induction of particular correlates of protective immunity.^{4,42-46} The nature of the early innate immune signatures has also recently been shown to be important in optimizing vaccine induced immunity.⁶ TLR agonists offer the most promising approaches to regulate DC activation and early innate immune signatures, by providing defined signaling mechanisms through known receptor mediated pathways.³ The TLR4 agonist MPL and the TLR9 agonist CpG are commonly used adjuvants in pre-clinical and clinical vaccine formulations for the induction of both cellular and humoral immune responses. We therefore sought to investigate their use in formulating our novel CMV vaccine which was based on CMV-poly-L and CMV-gB proteins. The CMVpoly-L protein includes 13 contiguous MHC class I-restricted CD8⁺ T cells from multiple CMV antigens and is designed to induce CD8⁺ T cell responses. The CMV-gB protein is an important source of CD4⁺ T cell responses and is also crucial target anti-viral neutralizing antibody responses. We believe that a bivalent vaccine based on these two proteins should provide an advantage over the previously designed subunit CMV vaccine formulations as our vaccine strategy will induce broad-based anti-viral immunity which is crucial for long-term protection.

Our initial immunization studies with the TLR4 agonist, MPL alone resulted in poor induction of innate immune gene signatures and reduced DC pro-inflammatory cytokine release. More importantly, CMV vaccine formulation with MPL alone was also inferior for the induction of anti-viral antibody, CD4⁺ and CD8⁺ T cell responses. In contrast the TLR9 agonist, CpG, induced an innate immune signature associated with the effective induction of anti-CMV CD8⁺ T cell immunity. This included genes known to play a pivotal role in innate cellular defense (*NOS2* and *OAS1b*), complement activation (*CIQB*), proinflammatory cytokines (*IL-6*, *IL-12b*, *IL-1A*, *IL-1B*, *CSFI*, and *CSF2*) cytokines (*IFNG* and *IL-18*), chemokines and chemokines receptors (*CCL3*, *CCL4* *CXCL10*, *CXCL11*, and *CCR5*), interferon regulatory factors (*IRF7*), intracellular adhesion molecule (*ICAM* and *CD38*), ISGylation (*ISG15*), and transcription factors (*JUN*). While our bivalent CMV vaccine formulated with CpG alone was sufficient to induce an optimal CD8⁺ T cell response, the addition of MPL significantly enhanced induction of CMV-gB specific CD4⁺ T cell responses and anti-viral neutralizing antibody immunity. These findings were consistent with our observations that the TLR4 agonist, MPL functions synergistically with CpG to enhance IL-12 production by PDCA1⁺ pDCs, and activate innate immune signatures which are crucial for induction of adaptive immunity. Earlier studies have shown that pDCs play a critical role in the induction of both cellular and humoral immune responses.^{47,48} Therefore, the synergistic effect of TLR4 and TLR9 on pDCs provides one potential mechanism

via which TLR4 further enhanced the immunogenicity of the bivalent CMV vaccine which includes both CMVpoly-L and CMV-gB proteins. Alternatively, the synergistic effects of TLR4 and TLR9 may be explained by other undefined mechanisms, such as changes in the recruitment of DC subsets to the DLN. Although MPL alone was unable to induce potent innate immune signatures, MPL alone or in combination with CpG triggered expansion in the numbers of pDCs and dermal DC subsets in the DLN. pDCs are uniquely able to produce large amounts of the antiviral cytokine (IFN- α/β) in response to nucleic acid sensing, which helps pDC to increase antigen presentation capacity and induction of innate and adaptive immune responses against viral antigens.^{49,50} In addition, although their contribution remains to be fully elucidated, recently dermal DCs have been shown to play a crucial role in activating the naïve CD4⁺ T cells.^{22,51} Based on these observations, we hypothesize that engagement of multiple DC subsets might have played a significant role in the induction of higher frequencies of CMV-gB specific CD4⁺ and CMVpoly-specific CD8⁺ T cell responses in mice immunized with bivalent CMV vaccine formulated with TLR4 and TLR9 agonists.

In summary, our findings provide a novel platform strategy for preventing CMV-associated disease/infection that maximizes humoral and cellular immunity using a protein-based vaccine with an adjuvant combination that can activate innate immune signatures, and recruit multiple resident and migratory DC subsets. More importantly, these observations may provide clues on future targeting of vaccine formulations for other persistent/chronic infections, for which broad-based immunity is essential.

Methods

Design of the expression vectors encoding CMV polyepitope protein

CMV polyepitope inserts were designed to encode multiple HLA class I restricted T-cell epitopes from three different antigens (pp65, IE-1, and pp50). The polyepitope sequences were designed in such a way that each epitope was linked to each other without a linking sequence (CMVpoly) or joined by a proteasome liberation amino acid sequence (AD or K or R) and a TAP transport motif (RIW, RQW, NIW, or NQY) (CMVpoly-L) (Fig. 1). 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 utilization and inserts were synthetically constructed (DNA2.0) and cloned into an expression plasmid (pJexpress 404) under an isopropyl- β -D-thiogalactopyranoside (IPTG) inducible promoter. These synthetically designed polyepitope constructs were transformed into chemically competent *E. coli* DH5 α (Invitrogen) and plasmids were purified using a QIAGEN maxi prep kit (QIAGEN).

Protein expression and purification

The expression and purification of the polyepitope protein was performed as previously described.^{52,53} Briefly, following transformation of the CMV polyepitope expression vectors (CMVpoly or

CMVpoly-L) into *E. coli* BL21 (DE3) pLysS (Invitrogen, Grand Island, NY), the expression of CMV polyepitope protein was induced for 4 h with 1 mM/mL of IPTG. The *E. coli* cultures were harvested by centrifugation, 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) and the CMV polyepitope proteins were purified using a 5 mL of Ni-NTA (QIAGEN) metal-affinity chromatography matrix. Following analysis of the eluted fractions using SDS-PAGE, positive fractions were pooled and protein was dialysed against 25 mM MES (2-[*N*-morpholino]ethanesulfonic acid) buffer pH 5.6. Protein concentration was determined using a Bradford assay kit (Bio-Rad).

Presentation of CD8⁺ T cell epitopes from CMV polyepitope protein by human cells

Epstein-Barr virus (EBV) transformed lymphoblastoid cell lines (LCLs) were pulsed with 25 μ g/ml of CMVpoly, CMVpoly-L, pp65, or IE-1 proteins for two hours at 37 °C, washed twice with RPMI 1640 medium, then resuspended in growth medium (RPMI 1640 with 10% FCS). After overnight incubation, LCLs were exposed to CMV-specific T cells at a responder to stimulator ratio of 4:1 for four hours at 37 °C; T cells were assessed for IFN- γ expression using an intracellular cytokine assay (ICS) as described previously.^{54,55}

Expansion of CMV specific T-cells from healthy donors PBMC

Peripheral blood mononuclear cells from healthy virus carriers were incubated with 25 μ g/ml of purified CMVpoly-L protein at 37 °C, 6.5% CO₂ for 2 h. CMV-poly-L pulsed PBMC were then mixed with autologous PBMC at a 1:1 ratio, and cultured in a 24 well plate for 10 d at 37 °C. Cultures were supplemented with recombinant IL-2 on days 3 and 6. T cell specificity was then assessed using an ICS assay.

Immunizations

HLA A2 transgenic mice (referred to as HHD-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, six-to-eight week old mice were immunized subcutaneously (s.c.) at the base of the tail with the CMV-gB (5 μ g/mouse) and CMVpoly-L (20 μ g) vaccine formulated with the TLR4 agonist (Monophosphoryl Lipid A (MPL; 25 μ g), alone, the TLR9 agonist CpG ODN1826; (CpG; 50 μ g) alone or with both the TLR4 and TLR9 agonists. The TLR agonists alone were used as a negative control. Mice were bled on day 21 prior to a booster dose. Mice were sacrificed on day 50 and assessed for both humoral and cellular immune responses. The TLR agonists were purchased from InvivoGen.

Intracellular cytokine staining to assess IFN- γ or multiple cytokine response

Following vaccination, isolated PBMC or splenocytes were cultured for 2 h with 10 μ g/ml of CMV-gB protein or 0.2 μ g of CMV peptides. Cells were then incubated overnight in the presence of GolgiPlug (BD PharMingen), washed twice, then incubated with APC-conjugated anti-CD3, FITC-conjugated anti-CD4 and PerCP conjugated anti-CD8. Cells were fixed

and permeabilised using a BD Cytofix/Cytoperm kit, then incubated with PE conjugated anti-IFN- γ . To assess the expression of multiple cytokines, cells were stained intracellularly with PE-conjugated anti-IFN- γ , PE-Cy7 conjugated anti-TNF and APC conjugated anti-IL2. Cells were acquired on a BD FACSCanto II and data was analyzed using FlowJo software. For this analysis, lymphocytes were first gated for expression of CD3 and then assessed for the expression of either CD4 or CD8. CD3⁺CD4⁺CD8⁻ or CD3⁺CD8⁺CD4⁻ T cell populations were then gated for the expression of IFN- γ , TNF, or IL-2. Polyfunctional cytokine expression was then determined using Boolean gate analysis.

Assessment of CMV-gB-specific antibody titers and avidity

Anti-CMV-gB antibody titers were determined using ELISA as previously described.⁵⁶ The isotypes of anti-CMV-gB antibodies in vaccinated mice serum samples were determined by ELISA using the mouse monoclonal antibody isotyping kit (Sigma). CMV-gB specific avidity was evaluated as previously described.⁵⁷

Micro-neutralization assay

The assay procedure was followed as described previously.⁵⁸ The percentage of neutralization of viral infectivity was calculated using the following formula: $([\text{number of IE1}^+ \text{ nuclei of CMV infected cells} - \text{number of IE1}^+ \text{ nuclei of serum treated CMV infected cells}] / \text{number of IE1}^+ \text{ nuclei of CMV infected cells}] \times 100)$.

RNA isolation and innate gene expression analysis

Inguinal lymph nodes were collected from HHD-I mice 24 or 48 h after vaccination with PBS, TLR4 alone, TLR9 alone, or TLR4 and TLR9, and single cell suspension was made using enzymatic digestion mix. Total RNA was purified using an RNeasy mini kit (QIAGEN GmbH) and the quality of RNA was determined using a ND-1000 spectrophotometer (Nanodrop Technologies). RNA was reverse transcribed using a High Capacity RNA-to-cDNA kit (Applied Biosystems). The Quantitative Real-Time PCR Gene Expression Assays for 92 genes were custom designed by Applied Biosystems. To further enrich gene specific targets, cDNA was preamplified using a pool of TaqMan Gene Expression Assays as a source of primers and TaqMan PreAmp kit (Applied Biosystems). Preamplified cDNA samples were loaded into Taqman Array 384 well Micro Fluidic Cards (Applied Biosystems) and sequences were amplified using the Applied Biosystems Viia 7 Real-Time PCR system. Raw data were obtained using SDS software, initially cycling threshold (Ct) values were normalized relative to the geometric mean of 3 housekeeping genes, *Actb* (β -actin), *Gusb* (β -glucuronidase), and *18s* rRNA. The quality control of all assays, relative quantification delta-delta Ct analysis; fold difference between the samples were determined using Ingenuity systems IPA software.

Murine dendritic cell subset analysis and impact of TLR agonists

Dendritic cells (DCs) were isolated from the spleens or inguinal lymph nodes of HHD-I mice by positive selection using CD11c MicroBeads (Miltenyi Biotech GmbH) and cultured in mouse DC growth medium (RPMI 1640 with 10% FCS, penicillin, streptomycin, non-essential amino acids, sodium pyruvate, L-glutamine, and β -mercaptoethanol) as described earlier.^{59,60}

For subset analysis, purified DCs were stained at 4 °C for 30 mins with different staining panels, including CD11c (AF700) (eBioscience), CD8 (PerCP) (BD Biosciences, New Jersey, MD), B220 (PE) (BD Biosciences), CD103 (FITC) (eBioscience), CD326 (PE-Cy7) (eBioscience), and DEC205 (APC) (eBioscience). Cells were washed and acquired on FACS Canto II to determine exact cell numbers.

To determine the inflammatory cytokine response, CD11c⁺DCs were incubated with MPL and/or CpG for 20 h. Supernatants were collected and levels of IL-6, IL-12p70, TNF, and IFN α were assessed using an ELISA assay (eBioscience). Expression of IL-12p70 in CD11c⁺ DCs was also analyzed by intracellular staining. Briefly, these cells were incubated with AF700-conjugated anti-CD11c and FITC-conjugated anti-PDCA-1 monoclonal antibodies (eBioscience), then fixed and permeabilised, and stained with PE-conjugated anti-IL-12p70 (BD Biosciences). Cells were acquired on a FACSCanto II and analyzed using FlowJo software.

Generation of VLE-specific T cell hybridoma

Spleen cells were collected from HHD-I mice immunized with an adenoviral vector encoding CMV epitopes¹⁶ were stimulated with irradiated, HLA A2-restricted IE-1 epitope VLEETSVM (referred to as VLE)-pulsed spleen cells in the presence of rIL-2. Fresh stimulator cells and rIL-2 were added every 7 d for 3 wk to generate VLE-specific murine CD8⁺ T cells. These VLE-specific CD8⁺ T cells were then fused with BWZ.36 cells and selected as previously described.^{61,62} BWZ.36 cells are stably transfected with NFAT-lacZ construct which is specifically induced in response to T-cell receptor interaction with peptide-MHC complexes on antigen presenting cells.^{61,62} The T cell hybridoma were then sub-cloned by limited dilution and CD3⁺ clones were selected to check VLE-specificity by evaluating T cell hybridoma activation after stimulation with VLE-pulsed MRC-5 cells as previously described.⁶³ Clone with CD3 expression and VLE-specificity were VLE-specific T cell hybridoma.

Cross-presentation analysis of CMV polyepitope protein by purified DCs

Enriched CD11c⁺ DCs from spleens were pulsed with or without 25 μ g/ml of CMVpoly-L protein, incubated for 2 h at 37 °C, then washed and resuspended in mouse DC growth medium containing TLR agonists. After overnight incubation, cells were exposed to a HLA A2-restricted VLE-specific murine T cell hybridoma cells for six hours and then cells were assessed for β -galactosidase (B-gal) expression using Fluorescein Di- β -D-Galactopyranoside (FDG) substrate.^{61,62}

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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