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Multi-antigenic human cytomegalovirus mRNA vaccines that elicit potent humoral and cell-mediated immunity

Shinu John, Olga Yuzhakov, Angela Woods, Jessica Deterling, Kimberly Hassett, Christine A. Shaw, Giuseppe Ciaramella *

Infectious Disease Therapeutic Area, Moderna, 500 Technology Square, Cambridge, MA 02139, USA

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ABSTRACT

A cytomegalovirus (CMV) vaccine that is effective at preventing congenital infection and reducing CMV disease in transplant patients remains a high priority as no approved vaccines exist. While the precise correlates of protection are unknown, neutralizing antibodies and antigen-specific T cells have been implicated in controlling infection. We demonstrate that the immunization of mice and nonhuman primates (NHPs) with lipid nanoparticles (LNP) encapsulating modified mRNA encoding CMV glycoproteins gB and pentameric complex (PC) elicit potent and durable neutralizing antibody titers. Since the protective correlates in pregnant women and transplant recipients may differ, we developed an additional mRNA vaccine expressing the immunodominant CMV T cell antigen pp65. Administration of pp65 vaccine with PC and gB elicited robust multi-antigenic T cell responses in mice. Our data demonstrate that mRNA/LNP is a versatile platform that enables the development of vaccination strategies that could prevent CMV infection and consequent disease in different target populations.

1. Introduction

Human cytomegalovirus (CMV) is a prototypical betaherpes virus that causes a panoply of clinical syndromes in at-risk populations [1]. CMV is the leading cause of congenital infection, resulting in severe abnormalities in infected newborns [2–6]. Additionally, CMV causes significant complications in immunocompromised individuals, particularly in transplant recipients [7,8]. Therefore, the development of a vaccine against CMV has been deemed a high priority by the Institute of Medicine [9], yet an effective vaccine against CMV remains elusive.

Neutralizing antibodies against envelope glycoproteins [10–13] and cellular responses targeting a variety of viral proteins, particularly pp65, IE1 and IE2 [14–16] have been implicated in controlling CMV infection and re-activation. The majority of the antibodies that neutralize infection of fibroblast cells is elicited by gB [17,18], whereas antibodies targeting the gH/gL/UL128/UL130/UL131A pentameric complex (PC) neutralize infection against endothelial, epithelial and myeloid cells [11,12,19–21]. The majority of neutralizing antibodies in CMV-seropositive individuals is directed against PC [12,19]. A recent Phase II trial with recombinant gB adjuvanted with MF59 showed only 50% efficacy in preventing primary infection [22,23]. Furthermore, a DNA vaccine expressing gB and pp65 [24,25], and alphavirus replicon particles (VRPs) encoding gB and pp65-IE1 fusion protein [26,27] demonstrated only modest potency in the clinic. These results highlight the importance of preventing infection in non-fibroblast cell types and reinforce the need for incorporation of PC for an optimally efficacious CMV vaccine.

Formation of PC is a multistep process involving assembly and proper folding of all five component proteins in the same cell and subsequent trafficking of the complex to the cell surface [28]. Absence or misfolding of any of the components will abolish PC formation [28]. Several experimental PC subunit vaccines have been generated using recombinant protein [29,30] and viral vector-based approaches [31,32]. Recently, a replication defective CMV vaccine based on the AD169 strain with restored PC was developed [33]. A major challenge with all these approaches is the requirement for maintenance of homogenous stocks of cell lines or viruses that can be scaled up to yield sufficient material of high purity for use in humans.

LNP delivery of modified mRNA encoding antigenic targets has emerged as a promising platform for vaccines and therapeutics [34–38]. A major advantage of the mRNA platform is the ability to potentially encode any antigen that can be rapidly synthesized and scaled up. Moreover, the platform enables delivery of multiple

* Corresponding author at: Moderna, 500 Technology Square, Cambridge, MA 02139, USA.
E-mail address: gciaramella@modernatx.com (G. Ciaramella).

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mRNAs encoding various antigens in a single immunization. Here we describe the generation of a functional PC utilizing modified mRNAs encoding the five different subunits of the complex. Encapsulation of all five PC mRNA constructs in LNPs allowed efficient delivery *in vivo* and generation of robust immune responses. Utilizing this mRNA/LNP platform, we developed a CMV vaccine expressing PC and gB that elicited potent and broadly neutralizing antibodies in mice and Non-Human Primates (NHP). Furthermore, we developed an additional pp65 mRNA/LNP that can be used in a heterologous prime/ boost vaccination regimen with PC and gB to broaden T cell responses.

2. Material and methods

2.1. Cells and virus

HEK293, HeLa, HEL 299, and ARPE-19 cells were obtained from American Type Culture Collection (ATCC). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. HUVEC cells (ATCC) were cultured in endothelial cell growth medium. CMV strain AD169 (ATCC) was propagated on MRC-5 cells and VR1814 (G. Gerna, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy) on HUVEC cells. Clarified supernatants were collected 10 days after 90% of cells showed cytopathic effect. Viral stocks were generated by adding FBS to a final concentration of 20%.

2.2. Western blot and immunoprecipitations

HEK293 cells were transiently transfected with mRNA encoding gH, gL, UL128, UL130, UL131A, or gB using Trans IT®-mRNA Transfection Kit (Mirus Bio LLC) per the manufacturer's recommendations. At 24 h post-transfection, cells were lysed in RIPA buffer (Boston BioProducts) supplemented with complete Mini, EDTA-free protease inhibitor cocktail tablets (ThermoFisher Scientific). Precleared lysates were resolved on Novex 4–12% Bis-Tris gels (Invitrogen) and blotted with rabbit polyclonal antibodies for gH, gL, UL128, UL130, or UL131A (D. Johnson, OHSU; Portland, OR) and mouse anti-β actin (Cell Signaling Technology). Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 680 goat anti-mouse IgG (ThermoFisher Scientific) were used as secondary antibodies. All images were captured on a ChemiDoc MP Imaging System (Bio-Rad Laboratories). For immunoprecipitations, lysates were first precleared with Protein G agarose beads (ThermoFisher Scientific), and gB was immunoprecipitated using an anti-gB monoclonal antibody (clone CH28, Santa Cruz Biotechnology). Immunoprecipitates were resolved on Novex 4–12% Bis-Tris gels (Invitrogen) and probed with mouse anti-gB antibody followed by incubation with HRP-conjugated rat anti-mouse IgG that recognizes native mouse IgG (Mouse TrueBlot® Western Blot Kit, Rockland Inc). Immunoblots were developed using TrueBlot substrate (Rockland Inc.) and visualized on a ChemiDoc MP Imaging System (Bio-Rad Laboratories, Inc.).

2.3. Flow cytometry

HeLa cells were transiently transfected with mRNA for the various subunits of CMV PC (gH/gL/UL128/UL130/UL131A) or combinations lacking one of the subunits or gB. After 24 h, the cells were harvested and resuspended in FACS buffer (1X PBS, 3% FBS, 0.05% sodium azide). To detect surface PC expression or components of PC, the cells were stained with human monoclonal antibodies 8I21 (PC), 3G16 (gH), 15D8var1 (UL128), and 7I13 (UL128/UL130/UL131A) in FACS buffer for 30 min on ice [12]. Thereafter, cells were washed twice in FACS buffer and incubated with Alexafluor 647 goat anti-human IgG (SouthernBiotech) in FACS buffer for 30 min on ice. All the above human monoclonal antibodies were custom synthesized by ThermoFisher from Expi293 cells that were transfected with expression plasmids encoding codon-optimized sequences for the respective heavy and light chain antibody. Surface gB was detected by mouse monoclonal anti-gB (clone CH28, Santa Cruz Biotechnology, Inc.). To detect intracellular gB, cells were permeabilized with 1X Cytofix/Cytoperm™ (BD Biosciences) and stained with mouse monoclonal anti-gB (clone CH28, Santa Cruz Biotechnology, Inc.). Alexafluor 647 goat anti-mouse IgG (SouthernBiotech) was used as secondary antibody. Surface staining was carried out as above. For intracellular staining, washing and staining was carried out in 1X Perm Wash buffer (BD Biosciences). The incubation time for the primary and secondary antibodies were 30 min on ice. Cells were acquired on a BD LSRII Fortessa instrument (BD Biosciences) and analyzed by FlowJo software v10 (Tree Star, Inc.)

2.4. Intracellular cytokine staining

Overlapping peptide libraries for gH, gL, UL128, UL130, UL131A (15-mer overlapping by 5 amino acids) and gB (15-mer overlapping by 11 amino acids) were synthesized by Genscript (Piscataway, NJ). A peptide library for PC was generated by pre-mixing the peptide pools for the five different components of the complex. The pp65 peptide library (15 mer overlapping by 11 amino acids) was from JPT Inc. Splenocytes were stimulated with peptides pools for PC, gB, and pp65 at 10 μg/ml for 5 h at 37 °C in the presence of BD GolgiStop™ and GolgiPlug™ (BD Biosciences) or left unstimulated. After 5 h, cells were surface-stained in FACS buffer in the presence of FcR blocking antibody 2.4G2 and eFluor™ 506 (eBioscience) as viability dye. Antibody clones used for surface staining were: anti-CD4 (GK1.5), anti-CD8 (53.6.7), anti-CD44 (IM7), anti-CD62L (MEL14), and anti-TCR β (H57-59). Intracellular staining was carried out with BD Cytofix/Cytoperm and BD Perm/Wash™ buffers (BD Biosciences). Surface and intracellular staining were carried out for 30 min on ice in FACS buffer and 1X Perm/Wash buffer, respectively. Antibody clones used for intracellular staining were: anti-IFN$_\gamma$ (XMG1.2), anti-IL2 (JES6-5H4) and anti-TNFα (MP6-XT22). Samples were acquired on BD LSRII Fortessa (BD Biosciences) and analyzed by FlowJo software (TreeStar, Inc.). Cytokine secreting T cells were plotted after background subtraction.

2.5. Generation of CMV modified mRNA vaccine constructs and formulations

Generation of mRNA encoding CMV antigens gH, gL, UL128, UL130, UL131A, and gB from strain Merlin was done by *in vitro* transcription using T7 polymerase from a linear DNA template that included 5′ and 3′ untranslated regions (UTRs) and a poly (A) tail as previously described [35]. mRNA encoding a phosphorylation mutant of pp65 (pp65 $^{\Delta P}$) was generated by deleting a.a 435–438 (RKRK). A pp65/IE1 fusion mRNA was constructed by assembling in tandem the sequences of pp65 gene lacking the stop codon with IE1 gene without the start codon to generate an in-frame fusion gene. S-adenosylmethionine was added to the methylated capped RNA (cap1) for increased mRNA translation efficiency. Similarly, a pp65$^{\Delta P}$-IE1 mRNA construct lacking a.a 435–438 of pp665 was also generated. LNPs were formulated as previously described [39]. Briefly, lipids were dissolved in ethanol at molar ratios of 50:10:38.5:1.5 (ionizable lipid:DSPC:cholesterol:PEG lipid). Two different LNPs having different ionizable lipids, referred to as LNP1 and LNP2, respectively, were developed. mRNA was combined with the lipid mixture, dialyzed and concentrated as previously described [35]. Empty LNPs lacking mRNA were also generated as controls. All formulations had particle sizes ranging

from 80 nm to 100 nm, with greater than 90% encapsulation and <1 EU/ml of endotoxin.

2.6. Mouse experiments

Six to eight-week old female BALB/c mice (Charles River Laboratories International, Inc.; Wilmington, MA) were immunized by intramuscular injection with 50 μl of the indicated LNP/mRNA formulations or empty LNP. All mouse studies were approved by the Animal Care and Use Committee at Moderna Therapeutics

2.7. NHP experiments

NHP studies were carried out at Southern Research Institute, Frederick, MD. Cynomolgous macaques (*Maccaca fascicularis*), hereafter Cynos, 2–5 years old and weighing 3–6 kg were immunized twice with varying doses of two different LNP formulations (LNP1 and LNP2) containing the mRNA constructs encoding CMV pentamer, gB, and pp65-IE1 antigens. Injections were given intramuscularly in a volume of 0.5 ml. All monkeys were screened for cyCMV and included in the study based on neutralization titers to CMV. The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Southern Research Institute.

2.8. ELISA

Overnight, 96-well microtiter plates were coated with 1 μg/ml of PC (Native Antigen Company) or gB (Sino Biological) protein. Serial dilutions of serum were added and bound antibody detected with HRP-conjugated goat anti-mouse IgG (Southern Biotech), followed by incubation with TMB substrate (KPL). The absorbance was measured at OD_{450nm}. Titers were determined using a four-parameter logistic curve fit in GraphPad Prism (GraphPad Software, Inc.) and defined as the reciprocal serum dilution at approximately OD_{450nm} = 0.6 (normalized to a standard on each plate).

2.9. Neutralization assays

Serum samples were heat inactivated at 56 °C for 30 min and diluted in complete medium. Cytogam was diluted to 10 mg/ml. Thereafter, samples were serially diluted in 2-fold steps and mixed with an equal volume of VR1814 or AD169 virus in serum-free media supplemented with 5% guinea pig complement (Cedarlane Laboratories Ltd) and incubated for 4 h at 37 °C, 5% CO2. The virus/serum mixture was then added to ARPE-19 or HEL299 cells in 96-well tissue culture plates and incubated for 17–20 h at 37 °C, 5% CO_2. Cells were fixed with 200 proof ethanol, blocked with superblock (Sigma-Aldrich), washed with PBS/0.05% Tween-20, and stained with mouse monoclonal antibody to CMV IE1 (Millipore), followed by Peroxidase AffiniPure Goat Anti-Mouse IgG (Jackson ImmunoResearch Laboratories) and developed with HistoMark® TrueBlue™ Peroxidase Substrate (SeraCare). CMV IE1-positive cells were counted using the CTL ImmunoSpot® Analyzer (Cellular Technology Limited). Neutralization titers (NT_{50}) were determined using a four-parameter logistic curve fit in GraphPad Prism (GraphPad Software, Inc.) and were defined as the reciprocal of the serum dilution resulting in 50% reduction in infected-cell count. In all experiments, the titers of Cytogam (CSL Behring) are shown for an approximate maximum concentration (2 mg/ml) in human sera after dosing, which was calculated based on an average body weight of 70 kg (assuming 5L of blood). CMV^+ sera was from Accuset™CMV Mixed Performance panel (SeraCare). This panel consists of undiluted plasma from 18 different individuals that were tested by the manufacturer for CMV IgM and IgG reactivity using commercially available anti-CMV assays. Wherever applicable, five samples that had the highest CMV IgG reactivity were selected for comparison.

2.10. Sera depletion assay

gH/gL protein was custom synthesized by ThermoFisher Scientific. Briefly, codon optimized sequences of truncated gH (715 amino acids) lacking the transmembrane and cytoplasmic tail and fused to a 6-histidine tag at the C terminus and full length gL were synthesized by GeneArt Gene Synthesis and subcloned into expression plasmids. The expression plasmids encoding truncated gH and full-length gL were co-transfected into Expi293 cells and the gH/gL protein dimer was purified by affinity chromatography on Ni-Hi Trap column (ThermoFisher Scientific). Purified gB and PC protein have been described previously. The correct conformation of the purified proteins was verified by ELISA using monoclonal antibodies TRL345 (gB), 8I21 (Pentamer) and 3G16 (gH).

Sera from immunized mice, Cynos, or Cytogam were heat inactivated at 56 °C for 30 min and incubated with and without purified gB, PC and gH/gL at a concentration of 50 or 100 μg/ml for 1 h at room temperature. Subsequently, the samples were tested for neutralization activity as described previously. To evaluate the specificity and efficiency of depletion, sera incubated with gB, PC, and gH/gL proteins were assayed in an ELISA with the respective proteins coated onto plates.

2.11. Statistical analysis

Data were analyzed with GraphPad Prism 7 (GraphPad Software, Inc.) using the Kruskal-Wallis test and Dunn's multiple comparison test or by two-tailed Mann-Whitney *U* test. A p value of <0.05 indicated statistically significant differences.

2.12. Data availability

All data are available upon reasonable request to the corresponding author. However, data on the composition of LNPs are proprietary and cannot be shared**.**

3. Results

3.1. High levels of surface PC expression in mRNA-transfected cells

We tested the expression of mRNA encoding the five subunits of the CMV PC by transfecting HEK293 cells with individual constructs. SDS-PAGE analysis revealed that all five mRNA constructs induced robust protein expression at predicted molecular weights (Fig. 1A). To determine the ability of these mRNAs to form a functional PC, HEK293 cells were co-transfected with all five subunit mRNAs in either equimolar or equal mass ratios. Cell surface expression of PC was analyzed by flow cytometry using a monoclonal antibody, 8I21, that recognizes a conformational epitope formed by four of the five subunits in the PC [12]. High levels of PC expression were observed, suggesting proper assembly and translocation of the multiprotein complex to the cell surface (Fig. 1B). Similar results were also observed in transfection of the PC components at equal mass ratios (Supplementary Fig. 1A and B). Lack of one of the PC subunits completely abolished formation of the complex (Fig. 1B) [12]. Furthermore, conformation-dependent neutralizing monoclonal antibodies against gH (3G16), UL128 (15D8var1) and UL128/UL130/UL131A (7I13) all bound to

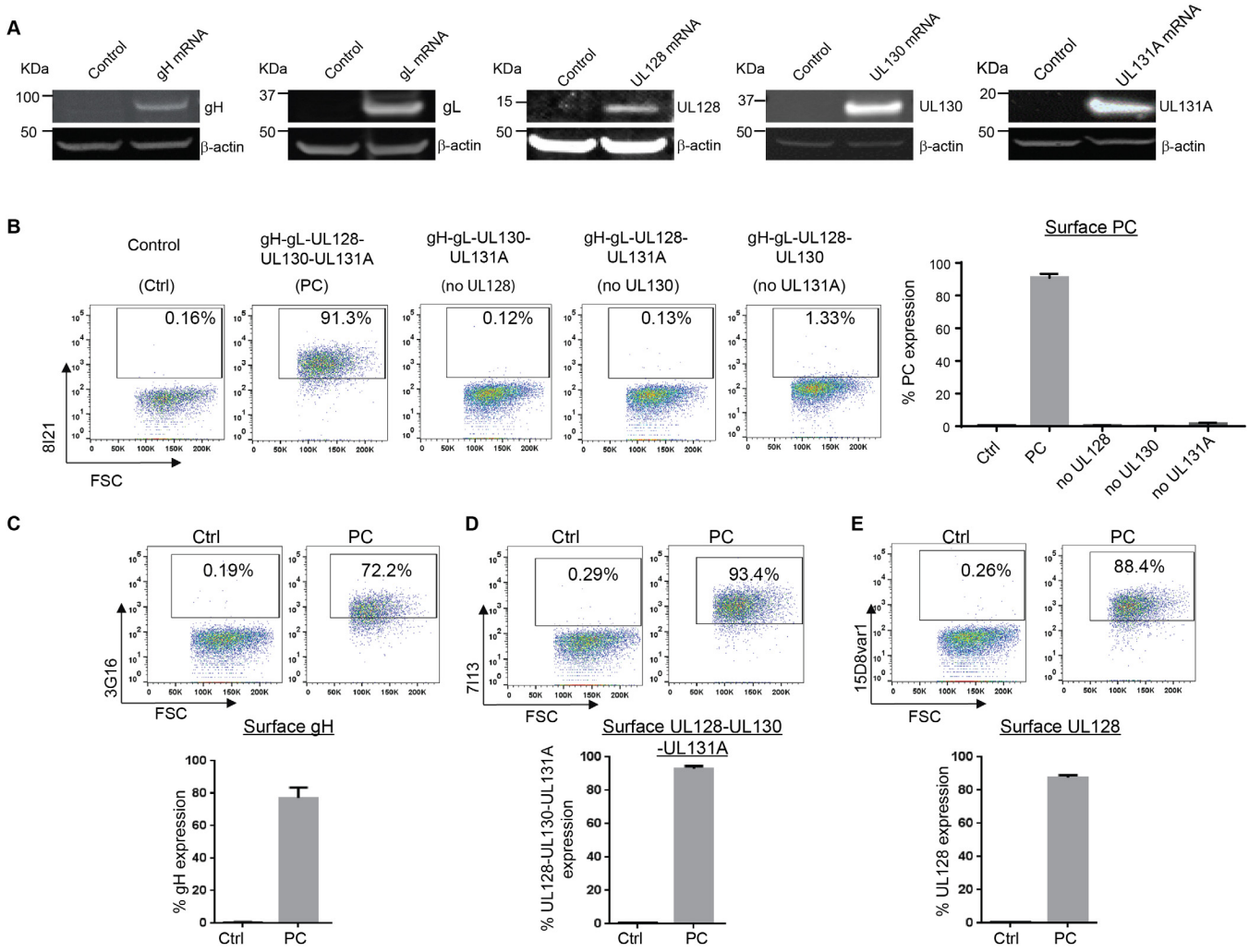

Fig. 1. Expression of PC subunit mRNAs and surface PC formation in cell lines. (A) Western blot analysis of gH, gL, UL128, UL130, UL131A, and β-actin in whole-cell lysates from HEK293 cells either untransfected (control) or transfected with the indicated PC mRNAs. Polyclonal antibodies against the various subunits were used for detection. β-actin served as the loading control. (B) Surface expression of PC. HeLa cells were transfected with mRNAs for all five subunits of the PC or lacking one of the subunits, as indicated. After 24 h, cells were stained with anti-PC antibody 8I21 and analyzed by flow cytometry. Representative flow cytometry plots (left) show PC surface expression. Bar graphs (right) show PC percent surface expression. See also related Supplementary Fig. 1A, B. (C–E) Flow cytometry analysis of expression of individual subunits or combination of subunits of cell-surface PC using indicated monoclonal antibodies. Representative flow cytometry plots (top) and bar graphs (bottom) depict percent cell-surface expression of the indicated subunits. Shown are representative data from one of two independent experiments. Data in bar graphs represent mean ± S.D.

surface expressed PC (Fig. 1C–F). These results further verified the proper folding of the mRNA expressed PC.

3.2. Intracellular and surface expression of gB

Next, we examined the expression of mRNA encoding full-length CMV gB in transfected HeLa cells. Intracellular and surface expression of gB was revealed by flow cytometry (Fig. 2A and B). Full-length gB exists as a 130 kDa precursor form and is cleaved by furin into an amino-terminal 115 kDa and a carboxy-terminal 55 kDa protein [40]. We tested if mRNA encoding gB underwent proteolytic processing by performing immunoprecipitations followed by SDS-PAGE analysis on lysates from HEK293 cell transfectants. A high molecular weight glycosylated form corresponding to 130 kDa and a faster migrating low molecular weight corresponding to 55 kDa could be detected, consistent with cleavage by endoproteases (Fig. 2C). The inability to detect the amino-terminal fragment suggested that the antibody recognizes an epitope within the carboxy-terminal fragment.

3.3. CMV mRNA vaccines elicit high titers of binding and neutralizing antibodies in mice

To assess immunogenicity of mRNA encoded CMV antigens, mice were immunized as shown in Fig. 3A with the indicated doses and groups of LNP/CMV mRNA constructs (Fig. 3A and B). Both PC and gB are targets of neutralizing antibodies, whereas pp65 and IE1 induce cell-mediated immune responses.

We first evaluated antibody responses to PC and gB by ELISA. Increases in antibody titers against both antigens were observed with increasing dose levels, which were boosted after a second or third dose of vaccine (Supplementary Fig. 2A and B). Importantly, the anti-PC and anti-gB antibody titers were not affected by the presence of the opposing antigen, suggesting a lack of interference by combining two different antigens in the same LNP formulation (Supplementary Fig. 2A and B).

We next evaluated the ability of these antibodies to block CMV infection of epithelial and fibroblast cells *in vitro*. Microneutralization assays showed potent and durable neutralizing antibodies

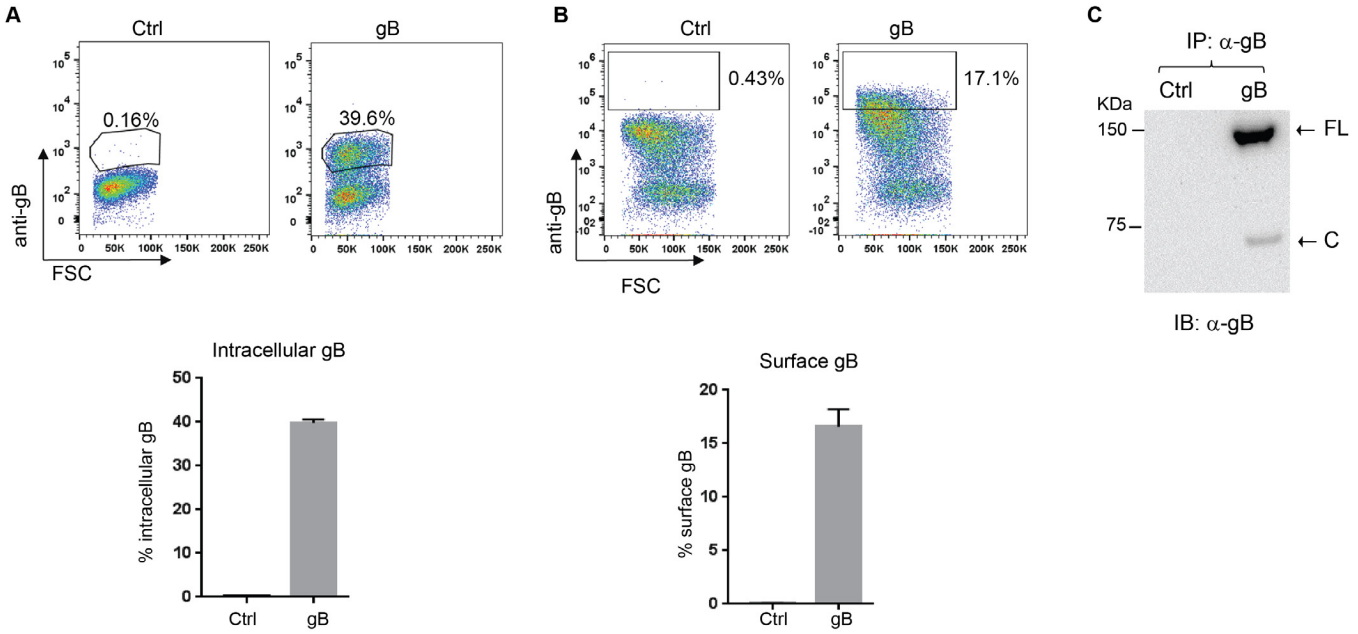

Fig. 2. *In Vitro* expression analysis of gB. (A, B) Intracellular and surface expression of full-length gB by flow cytometry. HeLa cells were either untransfected (control) or transfected with gB mRNA; after 24 h, the cells were either (A) fixed and permeabilized or (B) not fixed and stained with mouse monoclonal anti-gB antibody, and intracellular expression (A) and surface expression (B) were analyzed by flow cytometry. (A) Representative flow cytometry plots (top) and bar graphs (bottom) depict percent intracellular gB expression. (B) Same as (a) but showing surface expression. Shown are representative data from one of two independent experiments. (C) Immunoprecipitation of gB from lysates of HEK293 cells that were either untransfected (control) or transfected with gB mRNA. Detection of gB protein was done with anti-gB monoclonal antibody. Precursor (FL) and mature cleaved (C) forms of gB are depicted. Data in bar graphs represent mean ± S.D. Shown are representative data from one of two independent experiments.

against both cell types (Fig. 3B and C). To evaluate the potency of the neutralizing antibodies, we benchmarked to Cytogam, a hyperimmuneglobulin for CMV prophylaxis, and to sera from CMV$^+$ subjects. Our results showed that the neutralizing antibody titers were higher than or similar to Cytogam and CMV$^+$ donor sera for all vaccine groups tested at dose levels above 1 μg (Fig. 3B and C; Supplementary Fig. 2C and D).

To determine the specificity of antibodies that were generated with CMV mRNA vaccinations, mouse immune sera were incubated with purified gB, gH/gL, or PC proteins prior to performing microneutralization assays on epithelial or fibroblast cells. The correct conformation of the purified proteins (Supplementary Fig. 6A–C), the specificity and efficiency of depletion was determined by ELISA (Supplementary Fig. 7A and B). The neutralization activity against epithelial cell infection was completely blocked by purified PC but not by the other human CMV antigens tested (Fig. 3D). In fibroblast cells, neutralization activity of sera from mice immunized with PC + gB was partially completed by gH/gL and PC proteins but not by gB protein (Fig. 3E). This suggested that immunization with PC also generated anti gH/gL antibodies and that the same neutralizing epitopes in gH/gL are also exposed when gH/gL is part of PC.

3.4. CMV mRNA vaccine induces potent and durable neutralizing antibodies in NHPs

To investigate whether robust antibody titers are also generated in a larger animal species, we vaccinated Cynos using the dosing regimen shown in Fig. 4A with the indicated doses of two different cationic LNP (LNP1 [35,38] and LNP2) formulations containing the mRNA constructs encoding PC, gB, and a fusion of pp65 and IE1 (hereafter referred to as pp65-IE1) or a non-translating Factor IX mRNA (NTFIX) as a control (Fig. 4B). All monkeys used in this study had been naturally exposed to Cyno CMV (cyCMV) but had no neutralizing titers against fibroblast infection and the majority had low to undetectable neutralizing titers against epithelial (NT_{50} < 500) cells (day 0, Fig. 4B and C). ELISA results showed high titers of anti-PC and gB antibodies after vaccination (Supplementary Fig. 3A and B). A dose-dependent increase in neutralizing antibodies was observed three weeks following the second vaccination. The titers were higher than or equivalent to Cytogam and CMV seropositive sera in epithelial and fibroblast cells, respectively, at all doses of vaccine (Fig. 4B and C). Cynos that received a 100 μg dose were monitored for an additional six months following the second dose of vaccination. After the second dose, the neutralizing titers, initially dropped threefold against epithelial infection and three to fivefold against fibroblast infection but thereafter were sustained for an additional four months (Fig. 4B and C). The neutralizing titers elicited by the mRNA vaccine at 100 μg were also comparable to titers in CMV seropositive sera (Supplementary Fig. 3C and D). Overall, vaccination with mRNA formulated in LNP1 and LNP2 elicited equivalent neutralizing titers and therefore further studies utilized LNP2.

We further evaluated the specificity of these antibodies by performing antibody depletion experiments similar to those done with mouse immune sera. The specificity and depletion efficiency was determined by ELISA (Supplementary Fig. 7C and D). Purified PC and gB protein competed with neutralizing activity of NHP immune sera and Cytogam, in epithelial and fibroblast cells, respectively (Fig. 4D and E), consistent with published observations [41]. Altogether, our results demonstrate that vaccination with CMV mRNA antigens elicits antibody specificities similar to those observed in CMV seropositive individuals.

3.5. Strong T cell responses to pp65 are inhibited in the presence of other CMV antigens

T cell responses in CMV seropositive individuals predominantly target epitopes within the pp65 and IE1 antigens [42-44]. We

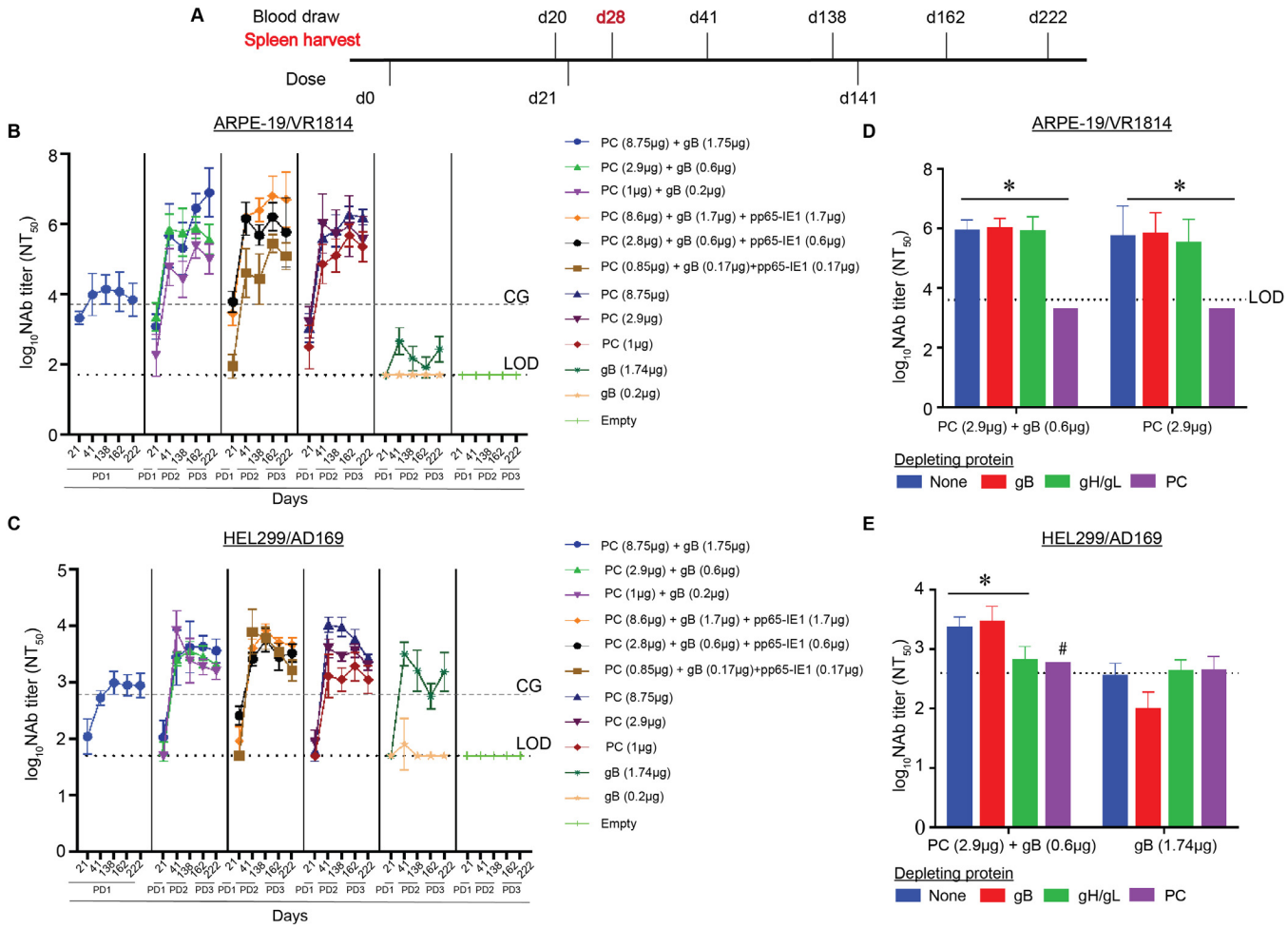

Fig. 3. Neutralizing antibodies and specificity of antibodies in mouse immune sera. (A) Schematic of vaccination regimen in mice. Indicated are days of dosing, blood draws, and spleen harvest. (B, C) Neutralizing titers in sera from mice immunized with the indicated doses and mRNA groups. All mRNAs were present at equal mass in the various vaccine groups. Numbers in parentheses depict the dose of each antigen. *PD1, PD2,* and *PD3* refer to postdose 1, postdose 2, and postdose 3, respectively. Shown are neutralization titers against (B) VR1814 infection in ARPE-19 epithelial cells and against (C) AD169 infection in HEL299 fibroblast cells. Symbols represent geometric mean titers (n = 5 for each group). (D, E) Specificity of neutralizing antibodies in sera of mice immunized with CMV mRNA vaccine. Mouse immune serum was preincubated with 50 μg/ml of purified gB, gH/gL, or PC protein prior to performance of neutralization assays. Shown are NT_{50} titers against (D) epithelial and (E) fibroblast cell infection. Each symbol represents an individual mouse. *LOD* refers to lower limit of detection and *CG* refers to Cytogam. *#:* Assigned an NT_{50} of 800, since spots could not be enumerated owing to background. All data are shown as mean ± S.D. n = 5 for each group. Statistical analysis was done using the Kruskal-Wallis test and Dunn's multiple comparison test ($^{*}p < 0.05$).

initially evaluated T cell responses to pp65-IE1 in mice using intracellular staining assay (ICS). Splenocytes were stimulated with peptide pools comprising select immunodominant peptides for pp65 and IE1 [26], and IFN_{γ}-producing T cells were measured by flow cytometry. In mice that were immunized only with pp65-IE1, IFN_{γ} production was detected in both CD4 and CD8 T cells (Fig. 5A and B). However, there was significant reduction in pp65-IE1 specific T cell responses when it was co-formulated with PC and gB mRNAs (Fig. 5A and B). To explore whether this phenomenon was unique to the pp65-IE1 fusion construct or also occurred with an established pp65 antigen, we synthesized an mRNA construct encoding phosphorylation mutant of pp65 ($pp65^{\Delta P}$) that retains immunogenicity but has reduced biologic activity [45]. First, we evaluated T cell responses to $pp65^{\Delta P}$ and compared it to pp65-IE1 immunization harboring the same mutation. Splenocytes were stimulated with overlapping peptide library for pp65 and IFN_{γ} producing CD4 and CD8 T cells evaluated by flow cytometry. The overall T cell responses were significantly lower in mice receiving $pp65^{\Delta P}$-IE1 as compared to $pp65^{\Delta P}$, indicating poor immunogenicity of the fusion mRNA construct (Supplementary Fig. 4A and B). Therefore, we proceeded to use $pp65^{\Delta P}$ (hereafter referred to as pp65) in our vaccine formulations.

Next, we asked if T cell responses to pp65 were also repressed in the presence of other CMV antigens. To address this, mice were immunized either with LNP encapsulating pp65 alone or pp65 + PC + gB. Splenocytes were stimulated with overlapping peptide libraries for pp65, and antigen-specific polyfunctional T cell responses were analyzed by ICS. Robust T cell responses were seen in mice immunized with pp65 alone; the majority of the T cells produced IFN_{γ} and TNF-α (Fig. 5C and D) and, to a lesser extent, IL-2 (Supplementary Fig. 5A). However, the pp65-specific T cell responses were significantly inhibited in the presence of other CMV antigens (Fig. 5C and D).

To determine whether pp65-specific T cell responses were repressed by other dominant antigens present in the multivalent vaccine, we evaluated antigen-specific T cell responses to PC and gB by ICS. Strong polyfunctional T cell responses to PC (Fig. 5E and F) and little to modest gB-specific T cell responses (Supplementary Fig. 5C and D) were observed. The majority of these PC-specific T cells secreted IFN_{γ} and TNFα (Fig. 5E and F) and, to a

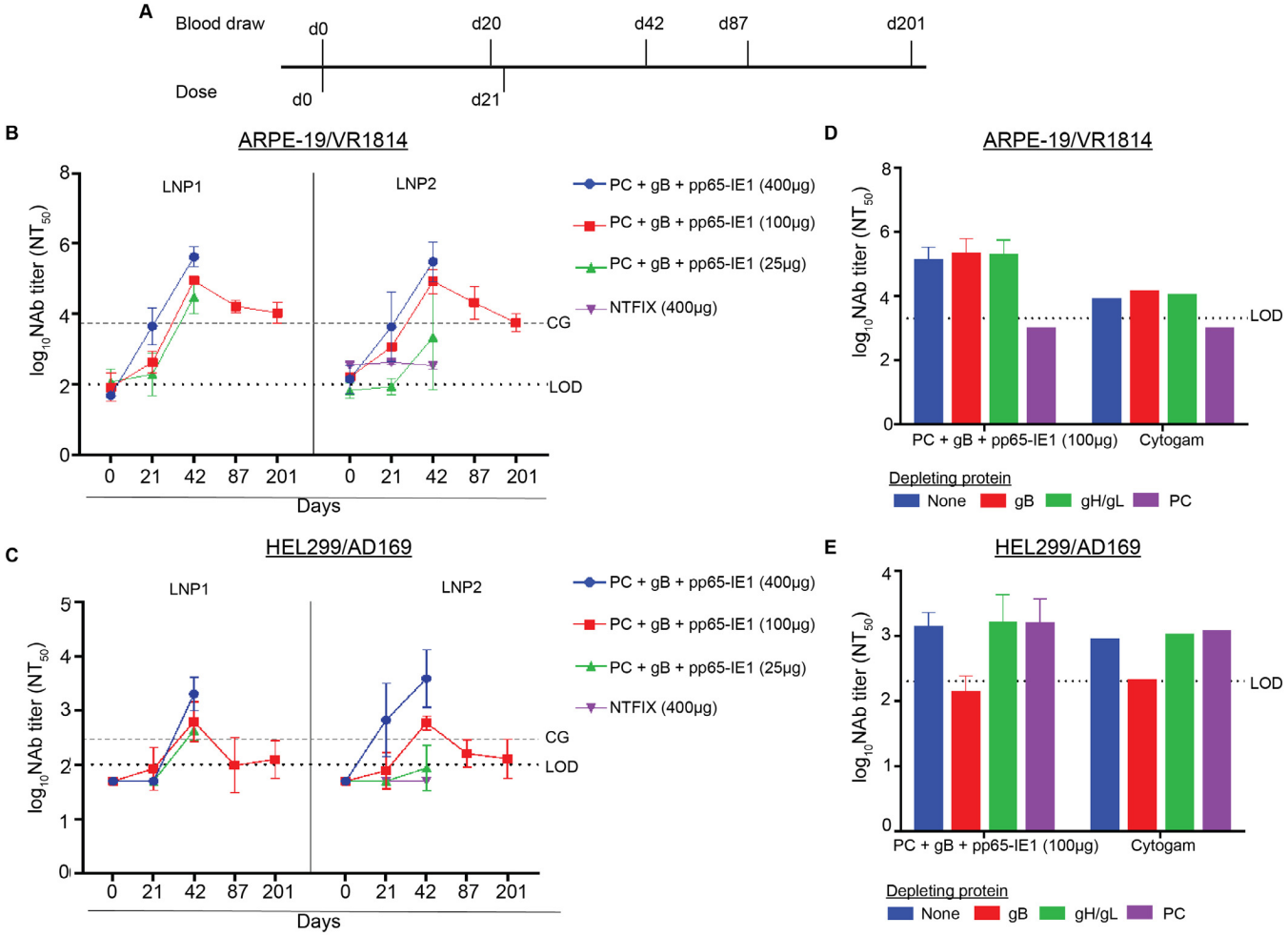

Fig. 4. Neutralizing activity and specificity of antibodies in sera of NHPs vaccinated with CMV mRNA vaccine. (A) Schematic of vaccination regimen and blood draws in NHPs. (B, C) Neutralizing titers measured as in Fig. 3 but in NHP sera that received two doses of the indicated vaccines. All mRNAs were present at equal mass in the various vaccine groups; the total dose is shown in parentheses. NT_{50} was measured on (B) ARPE-19 cells infected with VR1814 strain and (C) HEL 299 cells infected with AD169 strain. Each symbol represents a NHP (n = 3) (D, E) Specificity of antibodies elicited by immunization of NHPs with HCMV antigens. NHP immune serum and Cytogam were preincubated with 100 μg/ml of purified gB, g/gL, or PC protein prior to performing neutralization assays as described in Fig. 3. Shown are NT_{50} titers against (D) epithelial and (E) fibroblast cell infection. All results show mean ± S.D. Each symbol represents a NHP (n = 3) or mean from triplicates for CG. Statistical analysis was done using the Kruskal-Wallis test and Dunn's multiple comparison test ($^{*}p < 0.05$).

minor extent, IL-2 (Supplementary Fig. 5B). Altogether, our results suggest that the inhibition of T cell responses to pp65 stems from epitope competition due to dominating epitopes present in PC.

3.6. T cell responses to pp65 are restored by sequential immunization

We next addressed whether the issue of epitope competition could be resolved by a heterologous prime/boost regimen of a dose of LNP (pp65) followed by a dose of LNP (PC + gB + pp65). Control mice were immunized with a homologous prime/boost regimen of LNP (PC + gB + pp65) or a homologous prime/boost regimen of LNP (pp65) according to the dosing regimen shown in Fig. 6A. In mice that received a first dose of pp65 alone followed by all three antigens, pp65-specific CD4 and CD8 T cell responses were partially and fully restored, respectively, to levels observed in mice receiving two doses of pp65 alone (Fig. 6B and C). We further confirmed that a boost was necessary to induce robust pp65-specific T cell responses as a prime only vaccination with pp65 vaccine elicited low T cell responses (Supplementary Fig. 8). As expected, mice that received two doses of LNP (PC + gB + pp65) showed robust PC-specific T cell responses and negligible responses to pp65 (Fig. 6D and E). These results suggest that epitope competition can be resolved by a heterologous and sequential prime/boost vaccine regimen.

4. Discussion

PC-specific neutralizing antibodies are 100 to 1000-fold more potent than gH/gL and gB antibodies for neutralization in epithelial cells, endothelial cells, and monocytes [30]. Moreover, the presence of maternal antibodies to the PC early in pregnancy has been associated with a decreased risk of transmission to the fetus [46]. Therefore, the incorporation of PC in a CMV vaccine is highly desirable.

However, a major hurdle in the development of PC-based vaccines is the fact that the formation of PC requires co-expression and assembly of all five individual proteins in the same cell to form the multimeric protein complex, with all the neutralizing epitopes exposed [12].

We report here the development of a CMV vaccine utilizing modified mRNAs encoding the various subunits of PC or gB that are encapsulated into LNPs. The formation of PC in the native conformation was demonstrated by cell surface staining using an array of conformation-dependent monoclonal antibodies. Immunization

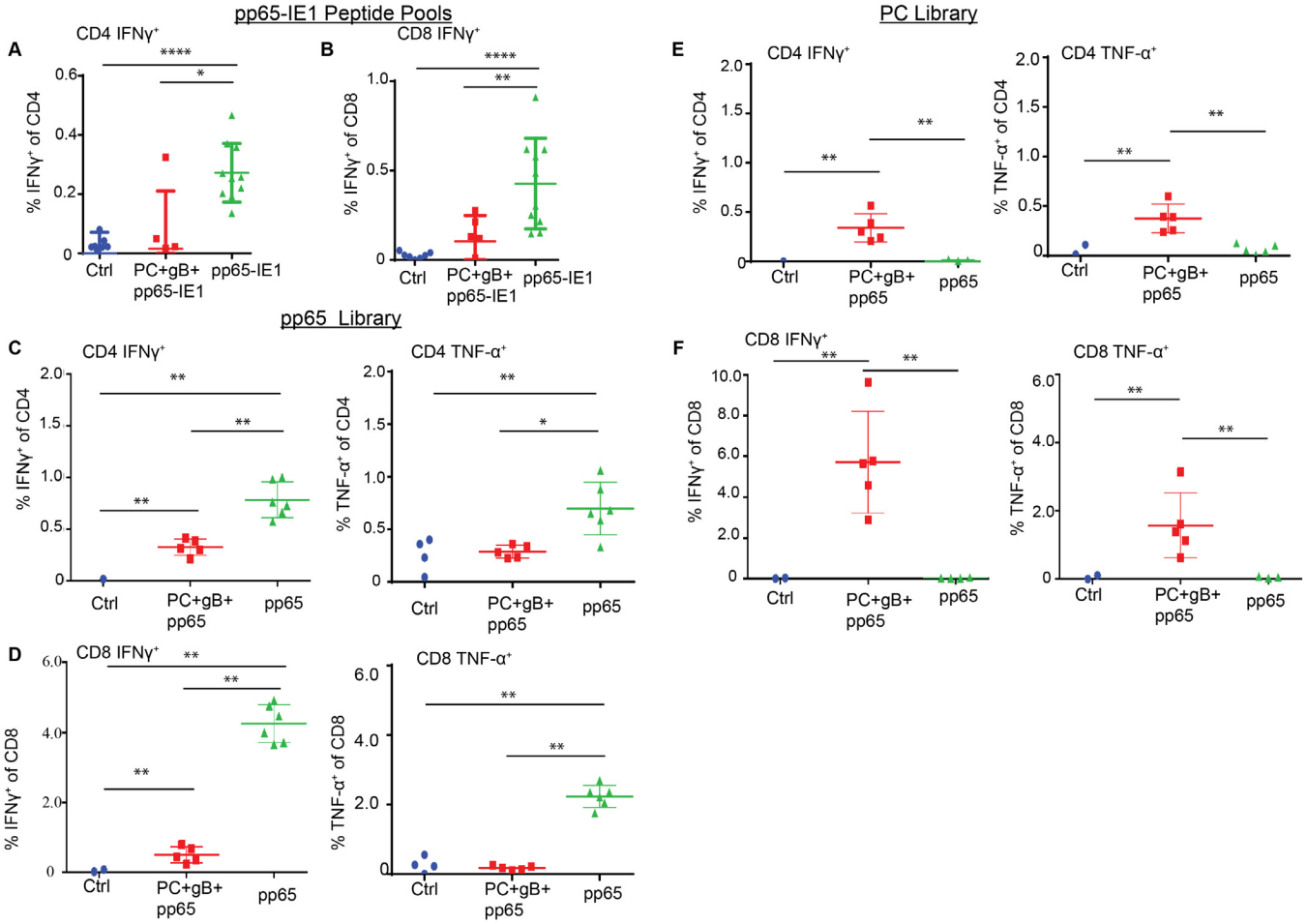

Fig. 5. Differential T cell responses to pp65 and PC in CMV mRNA vaccine. (A, B) T cell responses to pp65-IE1. One week following boost, CD4 (A) and CD8 (B) T cells secreting IFN_{γ} in response to pp65-IE1 peptide pools were measured by ICS and analyzed by flow cytometry. pp65-IE1 was present at a dose of 2 μg in both vaccine groups. See also Supplementary Fig. 4A, B. (C, D) pp65-specific CD4 (C) and CD8 (D) T cell responses. (E, F) PC-specific CD4 (E) and CD8 (F) T cell responses. One week postboost, splenocytes from the indicated groups were stimulated either with pp65 (C, D) or PC (E, F) peptide libraries, and polyfunctional (IFN_{γ}, TNF-α, IL-2) T cell responses were measured by ICS and analyzed by flow cytometry. Scatter plots represent mean ± S.D. For C–E, the doses of PC, gB, and pp65 were 8 μg, 2 μg, and 2 μg, respectively, wherever applicable. n = 5 for each group. Each symbol represents an individual mouse. Statistical analysis was done using the two-tailed Mann-Whitney *U* test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$). See also Supplementary Fig. 5A, B.

of mice and NHPs with LNP/mRNA vaccine elicited potent and durable neutralizing antibody titers against epithelial and fibroblast infection. In both animal species, the neutralizing antibodies peaked after the second dose and remained consistently above baseline, even after several months.

The neutralization activity in epithelial cells of sera from mice or NHPs immunized with PC or PC + gB was solely dependent on the PC and was significantly higher than the neutralizing activity in fibroblasts, consistent with published results [30]. In contrast, sera from mice immunized with PC or PC + gB neutralized fibroblast infection with comparable potencies. Since PC is not required for infection of fibroblast cells [28], it suggests that vaccination with PC mRNA generated both PC and gH/gL-specific antibodies. Similar results have been observed in previous studies using PC vaccines generated by other approaches [29–32]. Our studies demonstrated that immune sera from mice vaccinated with PC + gB were more potent at blocking viral entry in fibroblast cells than the sera from mice receiving gB alone. This is in agreement with observation from others [31] that in mouse immune sera, gH/gL antibodies were 10 to 20-fold more potent than gB antibodies at neutralizing fibroblast infection. In contrast, the neutralizing activity of NHP immune sera in fibroblast cells was entirely dependent on gB. The cause of the apparent discrepancy between the neutralizing activity of immune sera in fibroblast cells from mice and NHPs receiving the same mRNA vaccine antigens is unclear. It is possible that in NHPs, at least a fraction of the anti-gB antibodies are raised against domains of gB that contain neutralizing epitopes of higher potency, as seen in certain CMV seropositive individuals [47], that are better neutralizers.

T cell responses are also key in preventing CMV infection [48,49]. Our studies demonstrated that mice vaccinated with pp65 alone elicited robust polyfunctional T cell responses. Surprisingly, in mice that were immunized with PC + gB+pp65, we saw diminished T cell responses to pp65 and strong responses to PC. This was unexpected because PC is not known to elicit T cell responses following primary CMV infection in humans. Supporting this, examination of T cell responses against gH and gL in 17 healthy donors, found that only 20% of seropositive donors had a response to gH and gL with very few epitopes presented from these two glycoproteins, which were restricted to CD4 T cells [50]. In contrast, intramuscular vaccination followed by electroporation of mice with enhanced DNA (E-DNA) plasmid vectors encoding gH/gL or UL128/UL130/UL131A elicited robust CD8 T cell responses [51]. Our results are similar to those of the E-DNA vaccinations, but a direct comparison of the magnitude of T cell responses cannot be made due to the differences in the strains of mice and the assay

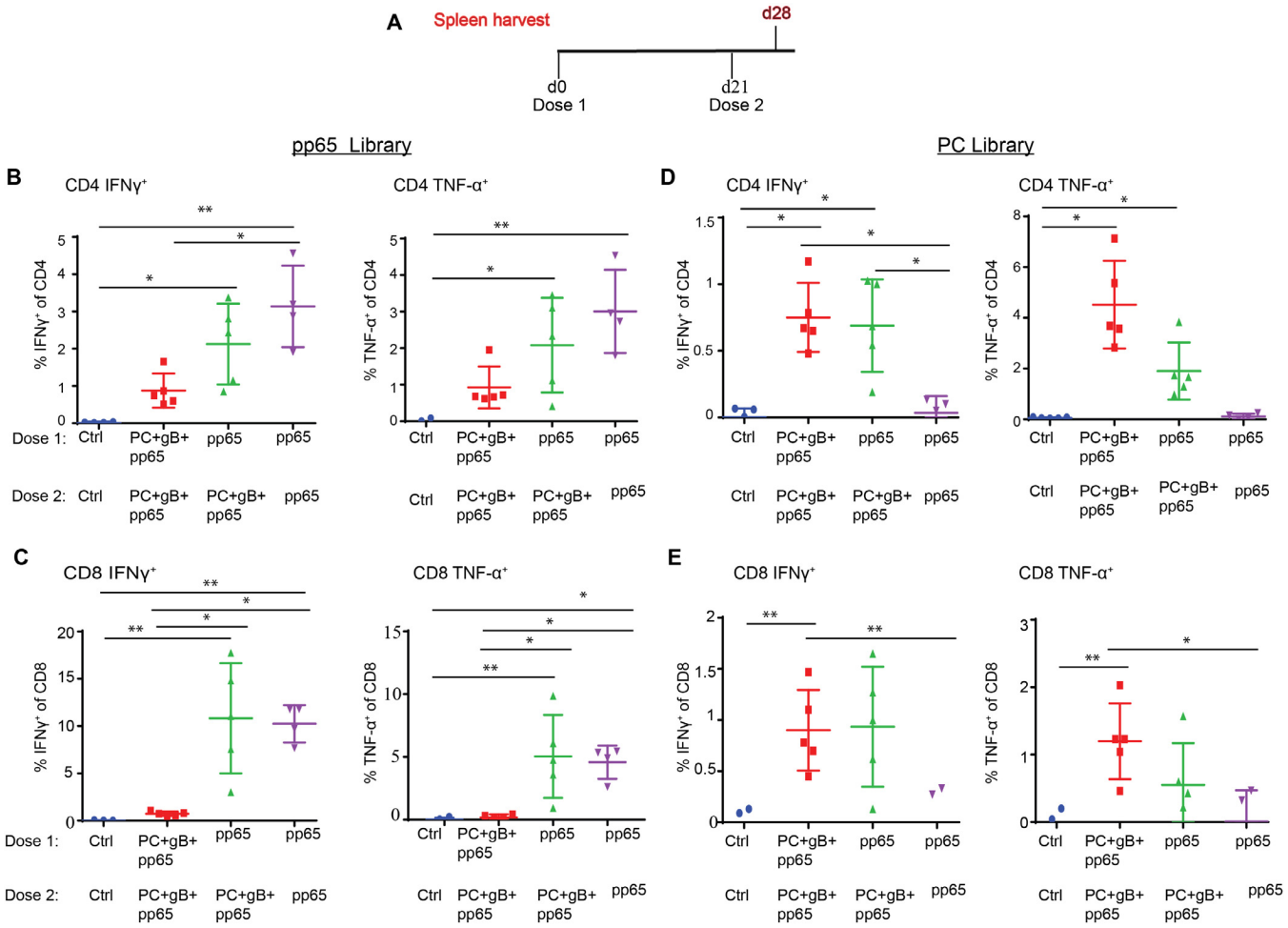

Fig. 6. Heterologous prime/boost vaccine regimen restores pp65-specific T cell responses. (A) Schematic of heterologous prime/boost dosing schedule (B–E) pp65-specific (B, C) and PC-specific (D, E) T cell responses in mice vaccinated with the indicated CMV mRNA antigens. Polyfunctional T cell responses were measured as described in Fig. 5. Shown are pp65-specific CD4 (B) and CD8 (C) and PC-specific CD4 (D) and CD8 (E) T cell responses. Scatter plots represent mean ± S.D. Each symbol represents an individual mouse. n = 5 for each group. Statistical analysis was done using the Kruskal-Wallis test and Dunn's multiple comparison test ($^{*}p < 0.05$, $^{**}p < 0.01$).

used to evaluate T cell responses. A possible explanation for the apparent discrepancy in T cell responses to PC between natural infection and vaccination could be due to differences in cellular uptake and antigen presentation and, potentially, in the lack of immunological repression in the nucleic acid vaccines, which is of course present during a natural infection.

Our observed immunodominance of PC-specific T cells in mice is intriguing in view of the fact that pp65 is a dominant T cell antigen and there is a high precursor frequency of pp65-specific T cells in CMV seropositive individuals [42,50]. Whether such immunodominance for PC-specific T cells also occurs in humans remains to be determined. The reduction in pp65-specific T cell responses, seen only in co-vaccination with PC, supports the notion that PC-specific T cells are potentially of higher affinity, dominating the primary and secondary immune response, thereby outcompeting the subdominant pp65-specific T cells.

There is compelling evidence that T cell competition, stemming from limited access to antigen presenting cells (APCs), is a key factor in determining immunodominance [52,53]. To alleviate competition for APCs, the subdominant epitope can be expressed prior to the dominant epitope, thus allowing enough time for the establishment of higher frequencies of T cells for the subdominant epitope. Indeed, our results demonstrate that a sequential vaccination of pp65 alone followed by PC + gB + pp65 restores pp65 T cell responses to levels that are comparable to vaccination with pp65 alone.

Several CMV experimental vaccines are either in development or in clinical trials to prevent CMV disease in transplant recipients. The majority of these vaccines have focused on T cell antigens alone, unfortunately with limited efficacy [54]. The addition of PC to a T cell antigen such as pp65 is likely to generate a more robust and balanced humoral and cell-mediated immune response, which may confer greater protection of fetuses as well as the transplant patients.

In summary, we have generated a CMV vaccine using a modified mRNA/LNP platform that elicits broad and durable neutralizing antibodies as well as robust T cell responses. To date, this is the only non live-virus-vaccine that includes the two dominant glycoproteins of CMV, namely gB and PC, as well as the immunodominant T cell antigen pp65.

Altogether our data show the versatility of the mRNA/LNP platform, which enables the rapid development of vaccines for complex multimeric antigens such as the PC and provides the flexibility to rapidly assess the most appropriate antigen combination to tailor the immune responses to target potentially different requirements, such as those that could be needed to effectively prevent CMV infection and/or disease during pregnancy or transplants.

Author's contributions

SJ and GC led the research. SJ, AW and OY performed experiments and analyzed data. CAS conducted statistical analysis. JD and KH made formulations. SJ wrote the paper. All authors reviewed this manuscript, revised it for important intellectual content and approved the final version of the manuscript for publication.

Conflict of interest

All authors are employees of Moderna.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.01.029.

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