Human cytomegalovirus pp65 peptide-induced autoantibodies cross-reacts with TAF9 protein and induces lupus-like autoimmunity in BALB/c mice

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Human cytomegalovirus (HCMV) has been linked to the triggering of systemic lupus erythematosus (SLE). We proposed that B cell epitope region of HCMV phosphoprotein 65 (HCMVpp65)422–439 mimics an endogenous antigen and initiates lupus-like autoimmunity. Amino acid homology between HCMVpp65422-439 and TAF9134-144 (TATA-box binding protein associated factor 9, TAF9) was investigated using a similarity search in NCBI protein BLAST program (BLASTP). A murine model was used to confirm their antigenicity and ability to induce lupus-like symptoms. HCMVpp65422-439 induced immune responses with the presence of specific antibodies against HCMVpp65422-439 and TAF9134-144, as well as anti-nuclear and anti-double-stranded (ds)DNA antibodies that are characteristic of SLE. In addition, the majority of HCMVpp65422-439 and TAF9134-144 immunized mice developed proteinuria, and their renal pathology revealed glomerulonephritis with typical abnormalities, such as mesangial hypercellularity and immune complex deposition. Immunoglobulin eluted from the glomeruli of HCMVpp65422-439 immunized mice showed cross-reactivity with TAF9134-144 and dsDNA. Increased anti-TAF9 antibody activity was also observed in the sera from SLE patients compared with healthy people and disease controls. Molecular mimicry between HCMVpp65 peptide and host protein has the potential to drive lupus-like autoimmunity. This proof-of-concept study highlights the mechanisms underlying the link between HCMV infection and the induction of SLE.

Systemic lupus erythematosus (SLE) is an idiopathic autoimmune disease characterized by the production of various antibodies against self-antigens, and tissue inflammatory responses that lead to severe organ damage. Infection and the subsequent immune response can result in persistent autoimmunity in genetically predisposed individuals. Human cytomegalovirus (HCMV) is a common pathogen, which is frequently seen in SLE and evokes disease via autoimmune-like components1. A possible mechanism that HCMV proteins share homologous sequence with the host and the cross-reactivity between viral and the host protein potentially contributes to the autoimmune response2, 3. The increased anti-HCMV IgM/IgG titer is often accompanied by the clinical and immunological presentation of SLE and has therefore been considered as a potential pathogenic agent in SLE4, 5.

Mouse model studies offer an understanding concerning the role of CMV in SLE. NZB/W F1 mouse, an F1 hybrid of New Zealand black female and New Zealand white male, and MRL/MpJ mouse with mutation of Fas gene (MRL/MpJ-Faslpr) are spontaneous models for SLE. The Th1-prone C57BL/6 and Th2-prone BALB/c are commonly used mouse strain for genetic and pathogenic investigation. Animal studies reported that CMV

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infection is linked with the development of SLE. For example, sialic acid-binding Ig-type lectin H (Siglec-H), a DA12-associated receptor on pDCs, modulates the secretion of IFN-α in plasmacytoid dendritic cells (pDCs)58. The elevated level of IFN-α in Siglec-H knockout (KO) mice did not promote the virus clearance after murine CMV (MCMV) infection but instead caused more severe lupus-like symptoms69. It has been demonstrated that MCMV neutralizing monoclonal antibody (mAb) cross-reacted with MCMV structural proteins and human nuclear U1 small nuclear ribonucleoprotein (U1 snRNP) autoantigen19. The administration of mice by this mAb led to edema of the hypodermis and mesangial proliferation in glomeruli19. Moreover, the M38 open reading frame (ORF) of MCMV is homologs to HCMV phosphoprotein 65 (HCMVpp65). Direct immunization of NZB/W F1 mice with plasmid DNA encoding MCMV M83 or HCMVpp65 proteins elicited early production of anti-dsDNA antibody and resulted in severe glomerulonephritis11.

HCMV phosphoprotein 65 (HCMVpp65) is a tegument protein found in abundance in the virion; it suppresses the host’s immune response by inhibiting expression of the host’s class II major histocompatibility (MHC) and attenuating interferon responses12–14. In healthy hosts, dominant T cell epitopes in HCMVpp65 elicit vigorous and specific cytotoxic T lymphocyte activity, whereas SLE patients tend to have a significantly higher prevalence of IgG antibodies against the HCMVpp65 protein compared with normal or other disease controls41,42. The B cell epitopes of HCMVpp65 (HCMVpp65336-561) have been fine-mapped to the hotspot region of HCMVpp65422-439 in our previous works43,44. The immunization of mice with either HCMVpp65336-439 or HCMVpp65422-439, together with murine C3d, has been shown to induce lupus-like autoantibodies and the subsequent development of autoimmunity43,44. At present, HCMVpp65428-437-GASTSTGKR is thought to be a critical epitope for provoking autoantibody production17.

The link between HCMVpp65 and the induction of SLE has rarely been investigated. A previous study by the authors revealed that animals who received the HCMVpp65422-439 epitope produced hallmark serological and renal features, which resembled human SLE17. The authors postulated that cross-reactive antibodies produced in TATA-box binding protein associated factor 9 (TAF9)134-144 or HCMVpp65428-439 immunized mice may lead to epitope spreading and contribute to the pathogenesis of glomerulonephritis. To verify this hypothesis, a murine model was designed, which immunized mice with a peptide-C3d complex with a streptavidin (SA)-biotin backbone that was shown to hasten the immunogenicity of the peptides. The presence of antibodies against HCMVpp65 and TAF9 proteins was also examined in human subjects with SLE, those with other autoimmune diseases, and normal controls to confirm the findings in humans.

Results

The correlation between anti-HCMVpp65422-439 and anti-TAF9 activities in SLE. The fine mapping of the linear B cell epitope within HCMVpp65422-439 has been performed in our previous works16,17. HCMVpp65428-437 is a dominant epitope to elicit IgG activity in mice at the early immunological stage. The targets of elicited IgG include HCMVpp65425-434, HCMVpp65428-437, and HCMVpp65430-439, which may be the result of epitope spreading17. The amino acid composition of HCMVpp65 peptides was showed in Fig. 1a. Therefore, we suppose that HCMVpp65428-437 is an immunodominant epitope of HCMVpp65422-439 to induce cross-reactive antibodies against viral and host proteins. To investigate whether the linear epitope, HCMVpp65428-437, was shared between HCMVpp65 and host proteins, a similarity search was conducted using the human amino acid sequence database. Five candidate proteins were identified, but the fragment of TATA-box binding protein associated factor 9 (TAF9)134-144 exhibited the highest alignment score, lowest E-value, and shared similar identity with HCMVpp65428-437 (70%, see Supplementary Table S1). We hypothesized that the sequence homology of HCMVpp65428-437 and host nuclear proteins might lead to the development of autoantibodies against TAF9 as a result of molecular mimicry.

We enrolled 67 SLE patients and 119 disease controls (primary Sjögren’s syndrome [SS], n = 23; rheumatoid arthritis [RA], n = 38; ankylosing spondylitis [AS], n = 18; gout, n = 40) and 72 healthy individuals. Their anti-HCMVpp65422-439 and anti-TAF9 IgG antibodies were examined to verify the association between anti-HCMVpp65422-439 and anti-TAF9 IgG antibody (Table 1). A significantly higher anti-HCMVpp65422-439 antibody titer was observed in SLE patients (0.706 ± 0.043) compared with individuals with SS (p < 0.001), RA (p < 0.001), AS (p = 0.049), gout (p < 0.001) and healthy controls (p < 0.001; Fig. 1b). This result is consistent with our previous findings17. Sera from patients with SLE exhibited a significantly higher titer of anti-TAF9 IgG antibody compared with the sera from patients with RA (p < 0.001), AS (p = 0.037), gout (p < 0.001) and healthy individuals (p < 0.001). No statistically significant difference was detected when the sera were compared with those from the SS cohort (p = 0.163; Fig. 1c). To confirm the ELISA results, sera (10 SLE, 1 RA, and one healthy control) that were dual positive for HCMVpp65422-439 and TAF9 were validated by western blot (Fig. 1d). In total, 9/10 SLE sera reacted to the full-length human TAF9 and HCMVpp65 proteins, but neither the normal nor RA sera exhibited a positive antibody response. The positive association between anti-TAF9 and anti-HCMVpp65422-439 antibody activities was significant in SLE, SS, and RA, however, most sera from SS, gout, or RA patients had a poor response to HCMVpp65422-439 or TAF9 in the ELISA test (Fig. 1e).

HCMVpp65422-439 and TAF9134-144 immunization induced cross-reactive antibodies to nuclear antigens and dsDNA. The role of the homologous sequence in the induction of cross-reactive antibodies against nuclear antigens was examined using a murine model, which immunized BALB/c mice with peptide antigens bound to maleimide-activated streptavidin (SA) complexed with four biotinylated C3d subunits (Fig. 2a,b)17. BALB/c mice immunized with HCMVpp65422-439-C3d or TAF9134-144-C3d (in the following referred to as HCMVpp65422-439 or TAF9134-144 immunized mice) had an elevated titer of anti-HCMVpp65422-439 or anti-TAF9134-144 IgG antibodies four weeks after immunization (Fig. 2c,d). Sera from HCMVpp65422-439 immunized mice recognized both peptides; however, TAF9134-144 immunized sera poorly reacted with HCMVpp65422-439 compared with the reactivity of HCMVpp65422-439 immunized sera against TAF9134-144.
The antinuclear antibody (ANA) and anti-dsDNA antibody are hallmark serological features of SLE. ELISA analysis and indirect immunofluorescence assay (IFA) were performed to determine the ANA profile in HCMVpp65422-439, and TAF9134-144 immunized mice. Immunization with HCMVpp65 422-439 or TAF9134-144 elicited an anti-HeLa IgG response, which began at four weeks and peak at 12 weeks after immunization (Fig. 2e).

Many different anti-nuclear antibody patterns can be identified in mice at 12 weeks after immunization with HCMVpp65422-439 and TAF9134-144, including nucleosome/chromatin, speckle/nuclear dots, MSA-II, centrioles, nuclear rim, and cytoplasmic proteins (see Supplementary Fig. S1 and Supplementary Table S2). Immunization with SA-C3d alone induced a low titer nuclear dots pattern, which was detected at 1:20 dilution. Nuclear staining

Figure 1. Association between anti-HCMVpp65422-439 and anti-TAF9 activities in SLE. (a) Alignment of the amino acid sequences for HCMVpp65422-439, HCMVpp65425-436, HCMVpp65428-437, HCMVpp65430-439 and TAF9134-144. B cell epitopes were recognized by purified anti-HCMVpp65422-439 IgG from the sera of human SLE, and HCMVpp65422-439 immunized mice, respectively. The TAF9 fragment contains a TAF9136-142 epitope like HCMVpp65428-437. (b,c) ELISA analysis for the detection of IgG against HCMVpp65422-439 and TAF9 protein using sera from patients with SLE (n = 67), SS (n = 23), RA (n = 38), AS (n = 18), gout (n = 40) and healthy controls (n = 72). (d) Western blotting for the examination of IgG antibodies response to HCMVpp65 and TAF9 proteins. There was a positive reaction to HCMVpp65 and TAF9 proteins using dual positive sera in the ELISA test. The positivity was defined as the mean ± 3SEM of the sera. Sera with an optical density (O.D.)450 > 0.932 of anti-TAF9 activity and O.D.450 > 0.835 of anti-HCMVpp65422-439 activity were considered to be dual positive sera. Asterisks indicate the sera are positive for HCMVpp65 or TAF9. SLE: sera of SLE patients; N and R: serum of normal population and patient with RA, respectively. P: positive control; His-tagged HCMVpp65 or TAF9 protein was recognized by the HRP-conjugated anti-His-tag antibody. Uncropped blots related to Fig. 1 were shown in Supplementary Fig. S4. (e) Correlation of anti-HCMVpp65422-439 and anti-TAF9 activities in patients with rheumatic diseases and healthy controls. Data are shown as the mean ± SEM of three independent experiments.
was not observed in SA-C3d mice at 1:100 dilutions or higher. Antibodies against cytoplasmic components were identified in HCMVpp65422-439 immunized mice (7/10), and TAF9134-144 immunized mice (2/10).

The presence of anti-dsDNA antibodies was tested using ELISA and C. luciliae assay. The semi-quantitative ELISA assay demonstrated that both HCMVpp65422-439 and TAF9134-144 immunized mice exhibited significantly higher titers of IgG against dsDNA compared with SA-C3d immunized mice four weeks after immunization (Fig. 2f). Three serial dilutions (1:20, 1:40 and 1:80) were further performed for the C. luciliae assay at 4, 8, 12, 14, 16 weeks after immunization (see supplementary Fig. S2a and supplementary Table S3). Anti-dsDNA activities were not found in SA-C3d immunized mice at dilutions of 1:40 or 1:80, while the HCMVpp65422-439 and TAF9134-144 immunized group had positive findings at four weeks at 1:40 (7/10, 5/10) and 1:80 (4/10, 2/10) dilutions, respectively. None of the mice with SA-C3d demonstrated any anti-dsDNA activity at 8–16 weeks after immunization. At 12 weeks after immunization, HCMVpp65422-439 immunized mice were observed a rise in anti-dsDNA level [1:20 (9/10), 1:40 (9/10) and 1:80 (8/10)]; TAF9134-144 immunized mice also resulted in anti-dsDNA serum activity [1:20 (8/10), 1:40 (7/10) and 1:80 (7/10)] after immunization. IgG1 and IgG3 were the dominant anti-dsDNA antibodies in both groups (Fig. 2g). The number of animals positive for dsDNA at 1:80 serum dilutions is reported in supplementary Fig. S2b. These results demonstrated that HCMVpp65422-439 and TAF9134-144 immunization induced antibodies reactive with cellular proteins and dsDNA.

Affinity Purified anti-HCMVpp65422-439 and anti-TAF9134-144 antibodies from sera of immunized mice and human SLE patients recognized dsDNA. To further examine the association of HCMVpp65422-439 and TAF9134-144 IgG antibodies, we performed the affinity chromatography to purify anti-HCMVpp65422-439, anti-TAF9134-144, and anti-TAF9 IgG antibodies from pooled sera of immunized mice at 10–12 weeks post-immunization and human SLE. ELISA analysis for anti-HCMVpp65422-439, anti-TAF9134-144, anti-TAF9, and anti-dsDNA activity was carried out using IgG eluted fraction. HCMVpp65422-439 and TAF9134-144 IgG antibodies either purified from human SLE or immunized mice reacted with HCMVpp65422-439, TAF9134-144, and dsDNA (Fig. 3a,b). Only a small amount of anti-TAF9 IgG antibodies were purified from mice sera. The mouse anti-TAF9 IgG antibodies had poor reactivity with HCMVpp65422-439, TAF9134-144, and dsDNA (Fig. 3a). In contrast, anti-TAF9 IgG antibodies purified from SLE sera reacted weakly with HCMVpp65422-439, TAF9134-144, and dsDNA (Fig. 3b).

For competitive ELISA analysis, HCMVpp65422-439, TAF9134-144, TAF9 or dsDNA as competitor agent was added respectively in each reaction well. In the animal study, the binding capacity of HCMVpp65422-439 and TAF9134-144 purified mouse IgG was inhibited or partially inhibited by HCMVpp65422-439, TAF9134-144, or dsDNA, but not by the TAF9 protein (Fig. 3c,d). The addition of HCMVpp65422-439, TAF9134-144, or dsDNA had no apparent suppressive effect on the anti-TAF9 activity (Fig. 3e). Similarly, in the human study, reactions between purified IgG against HCMVpp65422-439, TAF9134-144, or TAF9 and their respective targets were suppressed by HCMVpp65422-439, TAF9134-144, dsDNA, and TAF9. However, the competitive analysis of TAF9 in anti-TAF9134-144 activity was not significant (Fig. 3f,g). Interestingly, the partial suppression mediated by HCMVpp65422-439, TAF9134-144, and dsDNA were observed in anti-TAF9 antibody activities (Fig. 3b).

Immunization with HCMVpp65422-439 or TAF9134-144 induced early signs of SLE. The immunized mice were sacrificed at 16 weeks after immunization. Their kidneys were taken from immunized mice and examined for immune complex-mediated nephritis using immunofluorescence and hematoxylin & eosin staining. HCMVpp65422-439 or TAF9134-144 immunized mice had intense IgG (8/10 & 4/10), IgM (7/10 & 2/10) and C3 (4/10 & 3/10) deposition in their glomeruli, respectively (Fig. 4a). IgG1 (8/10 & 4/10) and IgG3 (8/10 & 4/10) were the most dominant in the glomerular deposits of HCMVpp65422-439 and TAF9134-144 immunized mice (Fig. 4a,b) and see Supplementary Table S4). No complement or Ig deposition staining was observed in the SA-C3d immunized mice. To evaluate the renal damage, the number of specific abnormalities in 100 glomeruli in a 5-μm-thick H&E-stained paraffin section from each kidney was recorded. The glomerular abnormality was scored in following categories: normal glomeruli (score 1), pure mesangial alterations (score 2), focal segmental glomerulonephritis (score 3), diffuse glomerulonephritis (score 4), Diffuse membranous glomerulonephritis (score 5) and advanced sclerosing glomerulonephritis (score 6), which are based on the 1982 classification published under the World Health Organization18. Mesangial alteration and moderate hyper-cellularity were observed in the glomeruli of HCMVpp65422-439, or TAF9134-144 immunized mice (Fig. 4c). The area of glomerular tuft and mesangial matrix were estimated by thirty HE-stained glomeruli from mice with the highest glomerulonephritis score in each group (Fig. 4d,e). The mice immunized with HCMVpp65422-439 exhibited a higher number of affected glomeruli and increased glomerulonephritis scores for renal lesions compared with TAF9134-144, or SA-C3d immunized mice (Fig. 4f,g). Urine protein analysis was performed at 4, 8, 12, 14, and 16 weeks after immunization.

**Table 1.** Characteristics of the enrolled study participants. RA, rheumatoid arthritis; AS, ankylosing spondylitis; SS, primary Sjögren’s syndrome; SLE, systemic lupus erythematosus.

| Characteristics | SLE | SS | RA | AS | Gout | Normal |
|-----------------|-----|----|----|----|------|--------|
| Age (years)     | 19-73 | 23-78 | 22-80 | 20-72 | 27-77 | 32-64 |
| Mean (years)    | 40.7±1.5 | 50.41±2.7 | 52.5±2.0 | 43.5±3.4 | 53.8±2.1 | 43.2±2.1 |
| Total no. of specimen | 67 | 23 | 38 | 18 | 40 | 72 |
| Female (%)      | 95.5% | 91.3% | 65.8% | 22.2% | 0% | 100% |


At the end of follow-up, proteinuria >100 mg/dL was observed in HCMVpp65422-439 immunized mice (6/10), and TAF9134-144 immunized mice (1/10).

To determine the binding activity of IgG deposited in glomeruli, glomerular isolation, and affinity purification of IgG from the glomeruli of HCMVpp65-422-439 or TAF9134-144-immunized mice was performed. The examination of anti-HCMVpp65-422-439, anti-TAF9134-144, anti-HeLa lysate, and anti-dsDNA activity from the sera of HCMVpp65, TAF9134-144, and SA-C3d-immunized mice was conducted using IgG fractions. As shown in western blot results, both eluted IgG fraction exhibited anti-HCMVpp65 and anti-TAF9 activities (Fig. 5a). The reactivity of eluted IgG against HCMVpp65422-439, TAF9134-144, or dsDNA was detected by ELISA test and C. luciliae assay (Fig. 5b,c). The eluted IgG from TAF9134-144 immunized mice showed the weak

(Fig. 4h).
antibody response to TAF9 protein. Notably, IgG1 and IgG3 were the major subclasses of anti-dsDNA IgG, which was found in human SLE sera as well (Fig. 5d). For the competitive assay, the ELISA analysis revealed that the partial repression mediated by HCMVpp65\textsubscript{422-439}, TAF9\textsubscript{134-144}, and dsDNA (but not by TAF9) was observed in anti-HCMVpp65\textsubscript{422-439} and anti-TAF9 IgG activity (Fig. 5e,f). To map the critical amino acid residues in TAF9\textsubscript{136-142}, seven glycine (G)- or alanine (A)- substituted peptides were used to measure antibody-binding capacity by ELISA test using eluted mouse IgG and purified anti-TAF9\textsubscript{134-144} IgG from SLE sera. We found that the IgG purified from human or eluted from glomeruli of mice recognized a similar epitope on TAF9\textsubscript{134-144} peptides (Fig. 5g). As the serine 136 or arginine 142 was replaced by glycine, an apparent reduction of anti-TAF9\textsubscript{134-144} activity was found in the ELISA test.

Figure 3. ELISA analysis for HCMVpp65\textsubscript{422-439}, TAF9\textsubscript{134-144}, and TAF9-specific IgG purified from pooled sera of immunized mice at 10-12 weeks after immunization or patients with SLE. ELISA analysis for anti-HCMVpp65\textsubscript{422-439}, anti-TAF9\textsubscript{134-144}, and anti-dsDNA activities using purified IgG from (a) immunized mice sera and (b) human SLE sera. A total of 1 μg anti-HCMVpp65\textsubscript{422-439} or anti-TAF9\textsubscript{134-144} IgG antibody, or 100 μl eluted anti-TAF9 IgG fraction (1 ml/tube) was used. ELISA competitive analysis for anti-HCMVpp65\textsubscript{422-439}, anti-TAF9\textsubscript{134-144}, and anti-TAF9 activities using purified IgG from the sera of (c–e) immunized mice sera and (f–h) human SLE sera for the competitive assay, 2 μg/well HCMVpp65\textsubscript{422-439}, TAF9\textsubscript{134-144}, dsDNA or TAF9 protein was used as competitor agents. Data are shown as the mean ± SEM of three independent experiments.
Discussion
The production of various autoantibodies is a hallmark of SLE. Molecular mimicry and epitope spreading are critical mechanisms underlying the development of autoimmunity. The current study used computational analysis to identify sequence homology between HCMVpp65_{422-439} and TAF9_{134-144} and SA-C3d immunized mice at 16 weeks after immunization. For this animal model, BALB/c mice were immunized with either HCMVpp65_{422-439} or TAF9_{134-144}, and then induced autoantibodies against HCMVpp65, TAF9, nuclear proteins, and SA-C3d. Immunoglobulin deposition was not found in glomeruli from SA-C3d immunized mice. Scale bar represents 100 nm. Glomerular tuft area. Glomerular hypertrophy is evaluated in three groups of mice. The ratio of expanded mesangial surface area to the total glomerular surface area. Glomerulonephritis score of renal lesions from immunized mice. Glomerulonephritis lesion counts. One hundred glomeruli per mouse were counted without overlapping. Ear holes produced by an ear punch device are used to identify individual mouse. L: left ear; R: right ear. Mice developed proteinuria at 4, 8, 12, 14, and 16 weeks after immunization. Data are shown as the mean ± SEM of three independent experiments. The full images related to Fig. 4 were shown in Supplementary Fig. S4.
and dsDNA was determined. The glomerulonephritis characteristics of SLE were also assessed. Although TAF9134-144 immunization induced a weaker humoral response compared with HCMVpp65422-439, IgG/C3 deposition and proteinuria were demonstrated in animals immunized by HCMVpp65422-439 or TAF9134-144. In addition, the competitive analysis revealed a positive association between peptide-induced antibodies and IgG deposition in the kidney glomeruli of mice. In the human study, both anti-HCMVpp65422-439 and anti-TAF9 autoantibodies were found in sera from patients with SLE. These results suggest that amino acid similarity between viral and host proteins promotes epitope spreading and accelerates the production of autoantibodies, which may, in turn, induce glomerulonephritis.

Several autoimmune disorders have been reported to link to the CMV infection, especially Sjögren's syndrome1,19. The characteristics of Sjögren's syndrome can be found in clinical diagnostics and experimental animals as a result of CMV infection that induces circulating autoantibodies against nuclear components and erythrocytes20,21. MCMV infection can induce anti-Ro/La antibodies and salivary gland inflammation in C57/BL6 lpr/lpr mice22. Likewise, HCMV infection elicits the expression of Ro antigen (60 KD/Ro, 52 KD/Ro) on the surface of keratinocyte and anti-phospholipid antibody production23–25. Also, HCMV early RNA has been demonstrated to induce antibodies against Ro antigen (SSA)24. In the current study, we enrolled patients with Sjögren's syndrome to determine the correlation between anti-HCMVpp65422-439, and anti-TAF9 antibodies. The ELISA analysis showed the low titer of anti-HCMVpp65422-439 antibody detected in patients with SS, suggested the epitope of HCMVpp65422-439 did not involve in the development of Sjögren's syndrome.

Many studies of HCMV have researched on the HCMV Towne and AD169 strains and focused on their potential host-virus interaction through efficient replication in human fibroblasts26,27. During HCMV infection, HCMVpp65 is transported into the nucleus mediated by two nuclear localization signals (HCMVpp65418-438 and HCMVpp65527-561) on C-terminus of HCMVpp6528. Another study revealed that HCMVpp65 binds to metaphase-arrested chromosomes in fibroblasts during infection, implying that HCMVpp65 does not merely bind to host proteins, but also forms immune-complex to nuclear components29. A similar finding was found in human polyomaviruses (HPyV) infection. The SV40 large T-antigen of HPyV forms complexes with nucleosome, subsequently is targeted by host immune responses, and elicits the generation of antibodies against both virus and

**Figure 5.** Identification of IgG antibody deposition in the glomeruli of immunized mice. (a) Western blot analysis of IgG eluted from the glomeruli of HCMVpp65422-439 and TAF9134-144 immunized mice against full-length HCMVpp65 or TAF9 protein. (b) ELISA analysis for anti-HCMVpp65422-439, anti-TAF9134-144, anti-TAF9, and anti-dsDNA activities using mouse IgG purified from glomerular deposition. 1 μg of eluted anti-HCMVpp65422-439 or TAF9134-144 IgG antibody was used for the test. (c) Representative images of *Crithidia luciliae* staining with 0.5 μg eluted IgG. Full images/blots were shown in Supplementary Fig. S4 (d) Isotypes of eluted IgG against dsDNA. Flow-through was used as the negative control. (e–f) ELISA competitive analysis for anti-HCMVpp65422-439 and anti-TAF9134-144 antibody activities using eluted IgG from the glomeruli of immunized mice. For the competitive assay, 2 μg/well of HCMVpp65422-439, TAF9134-144, dsDNA, or TAF9 protein was used as a competitor. (g) Mapping of critical amino acid residues on TAF9136-142 peptide. 1 μg of amino acid substituted synthetic peptides, and 1 μg eluted IgG antibodies from glomeruli of HCMVpp65422-439, or TAF9134-144 immunized mice, and IgG purified from SLE sera was used for the test. Data are shown as the mean ± SEM of three independent experiments.
host. These findings may provide the basis of hypothesis that the HCMVpp65 binds to immune complexes to form from genetic matrix or nuclear components, which may not merely be targets by anti-viral antibodies, but also raises the opportunity for B cell epitope spreading.

HCMVpp65 is an immunodominant T cell determinant in healthy individuals; however, the anti-HCMVpp65 antibody was more prevalent in SLE patients and had a higher specificity compared with other rheumatic diseases and healthy controls. The current study detected a significantly higher titer of anti-HCMVpp65 antibodies in the SLE cohort than normal cohort or diseased controls. Although some human subjects had autoantibodies against TAF9, it was not possible to detect antibodies reacting to TAF9 by western blot, suggesting that the detected antibody response in the ELISA test may be attributed to the conformation epitope of TAF9 being recognized by the tested sera. Western blot analysis found that 90% of the dual positive sera were positive for HCMVpp65 and TAF9 protein. The cross-reactivity between serum anti-HCMVpp65 and anti-TAF9 antibodies in SLE sera implied the possibility that the occurrence of the epitope is spreading between HCMVpp65 and TAF9 proteins during viral infection.

The poor immunogenicity of short peptides is a problem in peptide-induced immune responses, which often requires carrier proteins or molecular adjuvants to bridge innate and adaptive immunity. In the current study, the peptide-murine C3d complex was linked by a streptavidin-biotin backbone through the engagement of the C3d-CR2 and B cell receptor-peptides, which provided a stimulating signal to lower the threshold of T cell-dependent B cell activation. The immunization scheme of using a complex of streptavidin-conjugated HCMVpp65 and TAF9, with biotinylated C3d provoked a vigorous humoral-mediated immune response. Following immunization, HCMVpp65 and TAF9 immunization elicited antibodies against antigens from HeLa cells and produced ANA stain patterns. The production of multiple autoantibodies is a prominent feature of SLE. However, the humoral immune response was retained in mice 12 weeks after immunization, suggesting genetic predisposition plays a large role in SLE development.

Anti-dsDNA antibodies are a characteristic autoantibody for SLE, which plays a crucial role in lupus glomerulonephritis. Evidence for antigen selection of anti-dsDNA antibodies has illustrated that the basic or positively charged amino acid residues [arginine (R), asparagine (N) and lysine (K)], prefer to interact with DNA from virus or necrotic cell, and have the potential to induce anti-dsDNA antibodies during clonal expansion and affinity maturation. The ELISA test in the current study demonstrated anti-dsDNA reactivity in HCMVpp65 and TAF9 purified human antibodies and immunized serum. The HCMVpp65 and TAF9 immunization not only induced anti-dsDNA antibodies but also initiated early phase glomerulonephritis in BALB/c mice. In contrast, SA-C3d immunization did not induce ANA or dsDNA reactivity, which suggests that SA-C3d alone is less likely to induce autoimmunity.

Furthermore, TAF9 and HCMVpp65 specific IgG either from the immunized mice or SLE sera were predominantly made up of IgG1 and IgG3 isotypes. In human studies, IgG3 has been previously found to underlie autoimmune organ damage. Anti-glomerular basement membrane (GBM) IgG3 deposits along the GBM and tubular basement membrane plays a part in the renal injury of anti-GBM diseases. The formation and deposition of immune complex is a crucial factor exacerbating human lupus nephritis. The predominance of human IgG1 and IgG3 isotypes of anti-nucleohistone and anti-dsDNA antibodies was observed in the plasma of patients with SLE. In the current study, mice immunized with HCMVpp65 or TAF9 elicited serum titers of anti-dsDNA antibodies, which were positively correlated with the severity of IgG deposition and the severity of proteinuria (see supplementary Fig. S3 and Supplementary Table S5). In addition, IgG eluted from the glomeruli of immunized mice predominantly elicited IgG1/IgG3 antibodies against the immunizing peptide and dsDNA, suggesting that the pathogenic potential of anti-dsDNA antibodies may be involved in SLE.

The pathogenic role of anti-dsDNA antibodies in SLE has been extensively investigated. However, whether anti-dsDNA antibodies cause kidney damage remains controversial. Early studies indicated that nephritogenic mouse anti-dsDNA antibodies could interact with cell surface proteins on glomerular or vascular cells and lead to mesangial expansion and proteinuria. Recently, the deposition of positively charged nucleosomes and proteins on the GBM, have been described as targets for autoantibodies. The ELISA competitive analyses in the present study revealed that purified IgG from the kidneys of immunized mice recognized immunized peptides and dsDNA, which suggests that HCMVpp65 or TAF9 induced antibodies could contribute to IgG deposition on glomeruli.

In the ELISA competitive analysis, only a small amount of anti-TAF9 antibodies were purified from mice sera compared to antibodies purified from SLE sera (Fig. 3a,b). The purified anti-TAF9 antibodies either from mouse or human sera had low binding capacity for TAF9. We found that purified anti-TAF9 or anti-TAF9 antibodies from mouse or human sera were unable to react with (or be inhibited) by TAF9 or TAF9. To our best knowledge, the peptide/antigen coupled to CNBr-activated beads is one of the possibilities. For antibody purification, TAF9 (linear epitope) or TAF9 protein (conformational epitopes on the surface or structure of TAF9) is conjugated to CNBr activated beads in affinity column to selectively capture epitope-specific antibodies from sera. In the analysis of eluted IgG from glomeruli, we found that the eluted IgG from glomeruli of both immunized mice could react with full-length HCMVpp65 and TAF9 by western blot (Fig. 5a) but showed poor reaction with TAF9 in ELISA (Fig. 5c). We suggested that purified IgG antibodies against TAF9 and TAF9 have different epitope specificity on TAF9 protein.

On the other hand, seven amino acid-substituted synthetic peptides were used to examine the antibody activity with eluted IgG antibody from glomeruli of TAF9 immunized mice. The ELISA analysis revealed that the eluted IgG exhibited reduced antibody binding capacity for seven peptides. The polar (S137G) or positively charged amino acid (R142G) is more critical than nonpolar amino acid for antibody binding to peptides (Fig. 5g). However, this result cannot fully explain the mechanism of molecular mimicry in the current study, and more detailed experiments are needed for further validation.
In the current study, the immunization scheme was performed in a normal strain of mice, which were immunized by a C3d based adjuvant to enhance the antigenicity of the antigens; however, the humoral response was not sustained. This indicated that genetic factors play a pivotal role in the development or exacerbation of autoimmune diseases. The limitation of this study includes that our experimental design and data were unable to verify the existence of conformational epitopes due to the strategy of searching previously identified sequences, the method of IgG purification and the examination of IgG antibody responses.

**Conclusion**

The current study reports a positive association between anti-HCMVpp65_{422-439} and anti-TAF9 antibody reactivity in the sera of SLE patients. Immunization with the viral peptide-C3d complex provoked a strong humoral response in non-autoimmune-prone strains. The immunization of mice with HCMVpp65_{422-439} or TAF9_{134-144} induced cross-reactive antibodies and early signs of glomerulonephritis, which were characteristic of SLE. These results indicate that sequence homology between exogenous antigen and nuclear component is pivotal in the induction of SLE.

**Methods**

**Study populations.** All patients were recruited from rheumatology clinics in the Linkou branch of Chang Gung Memorial Hospital. The definition of SLE was based on the 1982 and 1997 American College of Rheumatology diagnostic criteria for SLE^{47,48}, and a rheumatologist confirmed all diagnoses. The study was approved by the Institutional Review Board of Chang Gung Memorial Hospital, and all patients provided their written informed consent before their inclusion as required by the Declaration of Helsinki (approval numbers #201600798B0 and 201600795B0).

**Bioinformatics analysis.** To determine the sequence homology of HCMVpp65_{428-437} peptides with nuclear proteins in humans, HCMVpp65_{28-437-GASTSAGKRR} was input as a query sequence using the BLASTP program in the alpha release 2.8.0+49,50. This procedure searched for high scoring sequence alignments between the query sequence and existing sequences in the reference protein database of Homo sapiens. Alignment score expect value and identity are the standard guidelines for evaluating the degree of homogeneity between sequences.

**Mice.** Normal 3 to 5-week-old female BALB/c mice were purchased from the National Laboratory Animal Center (NLAC), Taiwan. The mice were housed in a pathogen-free facility with an independent cage ventilation system; the laboratory animal experiment center was approved by the Institutional Review Board of the Chang Gung Medical Foundation (approval number #2017121309).

**Protein expression and purification.** The preparation of pET30a-HCMVpp65-C3d and pET30a-murine C3d constructs were as described previously^{17}. Plasmid encoding human TAF9 protein was purchased from Origene Technology (TAF9-RC201550, RefSeq: NM_003187.5), and the full sequences were amplified using TAF9 paired primers: (forward, 5′-CGGGATCCATGGAGTCGGGCAAGATGGCGCCGCT-3′; and reverse, 5′-CGGCTCGAGGAGATTATCAATGATCATCATC-3′). The PCR fragment was purified and ligated into TA cloning vectors (Yeasentar Biotech) to generate TA-TAF9. TAF9 gene fragment was then digested by BamHI/XhoI and ligated into pET30a. All the obtained pET30a constructs were confirmed via restriction enzyme digestion and DNA sequencing. Recombinant proteins were over-expressed in *Escherichia coli*, induced with one mM isopropyl−β-D-thiogalactoside (Sigma−Aldrich; Merck KGaA, Darmstadt, Germany) and purified by a nickel affinity column.

**Synthetic peptides and antigen preparation.** The preparation of synthetic peptides and antigens were followed according to previously described methods^{17}. The purity of all synthetic peptides was >99%, as per the manufacturers’ guarantee (GenScript Biotech Corp, New Jersey, USA). The peptides were prepared and stored according to the manufacturer’s recommendations (20 μg/μl). A total of 6 histidines and one cysteine were added to the C-terminus of the peptides as a target, or for crosslinking with the C3d protein. C3d biotinylation (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and maleimide activated streptavidin (Thermo Fisher Scientific, Inc.) conjugation were performed according to the manufacturer’s protocol. All complexes of peptide-C3d were generated and prepared for immunization within four hours before the injection to ensure the stability of the compounds.

**Immunization.** Female BALB/c mice (n = 25) were randomly separated into the following three groups: HCMVpp65_{422-439} (n = 10), TAF9_{134-144} (n = 10) and SA-C3d (n = 5). On day 1, the mice received an intraperitoneal injection with 100 μg HCMVpp65_{422-439}-C3d, TAF9_{134-144}-C3d or SA-C3d (emulsified with complete Freund’s adjuvant [Sigma−Aldrich]), respectively. Boosting was performed with complex antigens in incomplete Freund’s adjuvant (Sigma−Aldrich) on day 14, 28, and 42.

**Sera and urine collection.** The mice were bled from the retro-orbital vein sinus one day before each assay and at two-week intervals. Mouse plasma was collected from the blood by centrifugation at 4 °C for 10 minutes at 13,000 rpm. Unused plasma was stored at −80 °C, and the PBS-diluted sera were stored at 4 °C. Urine was collected to analyze proteinuria at the same time point. The course and onset of proteinuria were monitored at 4, 8, 12, 14, and 16 weeks after immunization by using proteinuria strip (Medi-Test Combi 10 VET strip, MACHEREY-NAGEL, Germany).

**ELISA analysis and immunoblotting.** Immunoblotting and ELISA were performed as previously described method^{17}. For the competitive assays, 2 μg/well HCMVpp65_{422-439}, TAF9_{134-144} dsDNA or TAF9 protein
Kidney tissues were collected and kept at 4 °C for antibody purification. The tissue slides were prepared for further investigation using coverslips with mounting medium (Diasorin). Sections were stained with FITC conjugated anti-mouse IgM/G and IgG antibodies (Jackson ImmunoResearch). Then, the protein G Sepharose beads were added to the HCMVpp65 422-439 or TAF9 134-144-CnBr gel, respectively and rolled at 4 °C overnight. Next, the Slurry of beads with CnBr was then added to the HCMVpp65 422-439 or TAF9 134-144-CnBr gel, respectively and rolled at 4 °C overnight. The protein G Sepharose beads were then washed three times with 0.1% NP40. The unbound portion of the sera or protein flow-through was collected and concentrated using a microcentrifugal concentrator (Sigma-Aldrich). Anti-nuclear antibody, and **Crithidia luciliae** immunofluorescence staining. The mice sera were tested for ANAs at 1:100 dilution in PBS using a standard ANA test (Diasorin).

**Isolation of the glomeruli.** The isolation of glomeruli was carried out using the modified protocol, according to Minoru Takemoto. Briefly, mice at 16 weeks post-immunization were anesthetized by an anesthetic machine vaporizer with isoflurane (3%) and perfused with 5 × 105 Dynabeads (4.5μm) in 40 ml of PBS through the heart. After mice sacrifice, their kidneys were immediately removed, cut into one-mm² pieces, and digested with collagenase (1.5 mg/ml collagenase A, 100 U/ml deoxyribonuclease I in Hanks' Balanced Salt Solution [HBSS]) at 37 °C for 30 minutes with gentle agitation. The collagenase digested tissue was gently pressed through a 70 μm cell strainer twice, and the cell strainer was then washed with 10 ml ice-cold HBSS. The filtered cells were then passed through a new cell strainer, and the cell strainer was washed with 10 ml HBSS. The cell suspension was centrifuged at 1,500 rpm at 4 °C for 10 minutes. The supernatant was discarded, and the cell pellet was re-suspended in 1.5 ml HBSS. Finally, glomeruli containing Dynabeads were harvested by a magnetic particle concentrator (Thermo Fisher Scientific, Inc.) for 20 minutes, and then washed at least three times with 5 ml ice-cold HBSS. Kidney tissues were collected and kept at 4 °C for antibody purification.

**Antibody purification.** The protocol for antibody purification was performed as previously described with small modifications. Moderated cyanogen bromide (CnBr) powder (Sigma-Aldrich) was activated according to the manufacturer's protocol. Briefly, 5 mg of 4 tandem repeats of the HCMVpp65 422-439 (GGGSGGGAAGGASTSAGRKRKRS) or TAF9 134-144 (GGGSKASTSAGRTRT) peptides were dissolved in a coupling buffer with activated CnBr gel and gently rotated at 4 °C overnight. The free active groups on CnBr were blocked by 0.1 M Tris-HCl (pH 8.0) at room temperature (RT) for 2 hours. The CnBr gel was washed with two cycles of alternating pH buffer (each cycle consists of a wash with pH 4.0 buffer containing 0.1 M acetic acid, 0.1 M sodium acetate, and 0.5 M NaCl followed by a wash with pH 8.0 buffer with 0.1 M Tris-HCl and 0.5 M NaCl) and once with 10 ml PBS. Then 300 μl of pooled HCMVpp65 422-439 or TAF9 134-144 mouse sera in 10 ml ice-cold PBS was then added to the HCMVpp65 422-439 or TAF9 134-144-CnBr gel, respectively and rolled at 4 °C overnight. Next, IgG was purified from the mouse glomeruli. Briefly, the glomeruli were homogenized by sonication with 6 M guanidine hydrochloride and 10-4 iodoacetamide. Dynabeads were removed from homogenized glomeruli by a magnetic particle concentrator, the flushed supernatant of homogenized glomeruli was incubated with protein G Sepharose beads and rolled at 4 °C overnight. The protein G beads were then washed three times with ice-cold HBSS and 0.1% NP40. The unbound portion of the sera or protein flow-through was collected and concentrated as the negative control. Bound antibodies were eluted by 1 ml, 0.1 M glycine (pH 2.0). The eluted samples were immediately neutralized with 50 μl neutralizing buffer (1 M Tris-HCl, 2 M NaCl, pH 8.8).

**Kidney immunofluorescence stain.** Kidney immunofluorescence stain was performed as previously described. Briefly, when the kidneys were removed from the mice, they were immediately placed in optimal cutting temperature gel, frozen with liquid nitrogen, and stored at − 80 °C before their use. The 3-μm-thick frozen sections were stained with FITC conjugated anti-mouse IgM/G and IgG antibodies (Jackson ImmunoResearch Laboratories) at a 1:100 dilution in PBS at RT for 30 minutes in the dark, humidified chamber. After washing, the tissue slides were prepared for further investigation using coverslips with mounting medium (Diasorin). After washing, the tissue slides were prepared for further investigation using coverslips with mounting medium (Diasorin).

**Hematoxylin and Eosin (H&E) staining.** Hematoxylin and eosin staining were carried out according to the Cold Spring Harbor protocols with small modification. The frozen sections of tissue and organs were immersed in 100% ethanol for 30 seconds after they were sectioned and then rinsed ten times in double-distilled H2O. Slides in the rack were then put into a container filled with hematoxylin for 5 minutes, and the rack was dipped five times into a jar containing 0.1% HCl. After dipping into tap water for 5 seconds, the rack was dipped into a jar containing 0.1% NH4OH 5 times and then into tap water five times. The cytoplasm was stained with eosin and dehydrated as follows: eosin for 3 minutes, 100% ethanol with 0.1% acetic acid for five dips, 100% ethanol I for five dips, 100% ethanol II for five dips, acetone I for five dips, acetone II for five dips, xylene I for five dips and xylene II for five dips. After removing any excess water, the slides were mounted and a cover glass was put on the slide for further investigation by microscopy (Olympus IX73/DP72, cellSens standard software). The
glomerular surface area and mesangial matrix area were estimated with thirty glomeruli of mouse with highest glomerulonephritis score in each group using NDP view 2 (Hamamatsu, Hamamatsu City, Japan).

**Statistical analysis.** The Student paired/unpaired t-test, and Fisher’s two-tailed exact test with graphs depicting the mean ± three standard error of the mean (SEM) was used for comparisons between 2 groups. Multiple comparison corrections and figures were performed using GraphPad Prism software 6.0. Pearson’s correlation coefficient test was used for these comparisons with graphs depicting the R squared and p-value. All tests of statistical hypothesis were done on the 2-sided 5% level of significance. Depending on the context, different levels of significance were reported (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001). All analyses were performed using SAS version 9.4 (SAS Institute).

**Ethics approval and consent to participate.** This study was approved by the Institutional Review Board of the Linkou Chang Gung Memorial Hospital (reference numbers 201600798B0 and 201600795B0). Written informed consent was obtained from all participants prior to sample collection. Animal experiments were approved by the Laboratory Animal Committee of the Linkou Chang Gung Memorial Hospital. All experiments were performed with full compliance with all relevant guidelines and regulations.

**Consent for publication.** The participants gave their written consent to the use of their clinical samples for data publication.

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Author contributions A.H.H. and C.F.K. designed the project. C.F.K., W.Y.T., Y.F.C., K.H.Y. and S.F.L. performed clinical diagnoses and acquired the data for analysis. A.H.H., Y.F.C. and C.F.K. performed the experiments, acquired the data and interpreted the results. A.H.H., Y.F.C., I.J.C., W.Y.T., K.H.Y. and S.F.L. participated in the interpretation of the results and drafted and revised the manuscript. All authors reviewed the manuscript, approved the final version to be published and agreed to be accountable for all aspects of the work.

Competing interests
The authors declare no competing interests.
