Immunophenotyping of Rhesus CMV-Specific CD8 T-Cell Populations

Nicholas L. Pomplun,1† Logan Vosler,1† Kim L. Weisgrau,1 Jessica Furlott,1 Andrea M. Weiler,1 Hadia M. Abdelaal,2 David T. Evans,1 David I. Watkins,3 Tetsuro Matano,4 Pamela J. Skinner,2 Thomas C. Friedrich,1 Eva G. Rakasz1*

1Wisconsin National Primate Research Center, University of Wisconsin-Madison, Madison, Wisconsin
2Department of Veterinary and Biomedical Sciences, University of Minnesota, Minneapolis, Minnesota
3Department of Pathology, Miller School of Medicine, University of Miami, Miami, Florida
4AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan

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†These authors contributed equally to this study.

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As a causative agent of considerable morbidity and mortality, human cytomegalovirus (hCMV) has been included among the high priority targets for therapeutic vaccine development since the year 2000 (1-3). hCMV belongs to the Betaherpesvirinae subfamily of Herpesviruses. Having been around for more than 400 million years (4-6), Herpesviruses co-evolved with the immune system of their hosts. As a result, they acquired the ability to establish life-long, productive infection in the presence of abundant cellular and antibody responses. Cytomegaloviruses are highly species-specific pathogens, infecting a wide range of hosts from rodents to primates (7). Primate CMVs are more similar to each other than to CMV in rodents. Cross-species infection is possible, but rare and it mainly occurs between highly related species, such as rhesus and cynomolgus macaques (8, 9). Nonhuman primates represent an invaluable intermediate step in translating vaccination concepts from murine models to humans. Therefore, a thorough understanding of the virus-host interaction between CMVs and nonhuman primates is essential.

Captive-bred rhesus macaques (Macaca mulatta) become naturally infected with CMV during the first years of their life. Based on rhCMV-specific IgM and IgG antibody data, seroprevalence can reach up to 100% (10-12). Although the virus is
present in the breast milk, transmission with nursing appears to be less efficient than in human, since seroconversion usually happens well after weaning, mostly following the animals’ introduction into the general colony (13). Cellular immune responses can be detected at very low levels as early as six months of age. Circulating T-cell responses are readily detectable in the magnitude of 10^{5} SFU/million PBMC against viral proteins IE-1, IE-2, or pp65 with IFN-γ elispot in adult animals (13). However, in the absence of basic tools, such as rhCMV-specific tetramers, accurate monitoring of these responses cannot be accomplished.

Therefore, our goal in the current study was to define CD8 T-cell epitopes in the viral proteins immediate early protein-1 (IE-1), and phosphoprotein 65-2 (pp65-2). Since tetramers binding to T cells that recognize the human CMV orthologs of these proteins already exist (14, 15), rhCMV-specific tetramers with similar targets will enable the research community to perform direct comparison between the two species.

MATERIALS AND METHODS

Animals and Tissue Preparation

We obtained samples from 67, more than three-year old rhesus macaques of Indian origin, housed at the Wisconsin National Primate Research Center (WNPRC). All macaques were cared for in accordance with the guidelines of the Weatherall report and the principles described in the US National Research Council’s Guide for the Care and Use of Laboratory Animals, and the US Public Health Service’s Policy on Humane Care and Use of Laboratory Animals. The animal welfare assurance no. of WNPRC is A3368-01. The macaques were assigned to various experimental protocols, each approved by the University of Wisconsin Graduate School Animal Care and Use Committee. The animals’ MHC-I genotype was determined by the Genetics Services core laboratory of the WNPRC using either sequence-specific PCR analysis or amplicon deep sequencing as described elsewhere (16, 17). Animals were perfused with heparin-containing PBS before tissue harvesting to minimize blood contamination in richly vascularized organs like the lung and kidney. Whole lymph nodes, a 1 cm × 2 cm portion of the spleen and the salivary gland were cut into 1 mm × 1 mm pieces in sterile RPMI 1,640 and pressed through a 70 μm cell-strainer to remove cellular debris. Strained spleen lymphocytes were purified by Ficoll-Paque PLUS (GE Healthcare Systems, Uppsala, Sweden) density centrifugation. An approximately 1 cm × 2 cm piece of the lung, the kidney, half of the urinary bladder, and a 5–6 cm-long section of the aorta and the vena cava were cut into small pieces and digested in R10 containing 400 U/ml collagenase Type I, 120 U/ml collagenase Type XI, 60 U/ml hyaluronidase, and 60 U/ml DNase1 at 37°C for one hour, then filtered through a 70 μm cell-strainer to remove cellular debris. Cells were counted and further processed for flow cytometry and viral load quantification assays.

Peptides

15-mers overlapping by 11 amino acids covering the entire length of rhCMV strain 68-1 proteins pp65-2 (18) and IE-1 (19) were synthesized using the services of GenScript Biotech Corp (Piscataway, NJ). GenBank accession numbers are: M93360 for IE-1 (19) and AY186194 for the whole rhCMV 68-1 genome (20). Peptides between 7 and 14 amino acid-long were obtained from the same source and used to define the minimal optimal epitopes. Lyophilized peptides were dissolved in 100% DMSO (Sigma) to create stock solutions of 40 mg/ml concentration. Stock solutions were diluted with sterile HBSS to create 4 mg/ml (100×) working solutions. All stocks were stored at −20°C.

rhCMV Epitope Definition with Elispots

We used freshly isolated or cryo-stored PBMC from EDTA-anticoagulated blood samples in the initial screenings. We detected IFN-γ secreting cells using ELISPOT PLUS kits (MABTECH Inc, Cincinnati, OH) according to the manufacturer recommendations. We used stimulation conditions and analysis criteria as described previously (21). Briefly, 1–2.5 × 10^{5} PBMC/well were stimulated with pools of peptides containing 10 adjacent overlapping 15-mers at approximately 2.2–2.6 μM concentration. Wells were imaged and spot-forming cells (SFCs) counted with an AID ELISPOT reader (AID, Strassberg, Germany). Results were considered positive when the mean number of SFCs from duplicate test wells was greater than two times the mean plus two standard deviation of the background SFCs (duplicate wells with no peptide introduced). Positive responses were >50 spots/million cells. Cells stimulated with 10 μg/ml Concanavalin A (Sigma, Aldrich) were used as positive controls. To determine the phenotype of IFN-γ secreting populations we performed ICS using the positive pools as stimulation. The pools were deconvoluted with elispot to identify the stimulating 15-mers, if the responding cells were CD8 positive. 15-mers were serially truncated to unequivocally define the minimal optimal epitope (MOE).

rhCMV-Specific Primary CD8 T-Cell Lines

We generated rhCMV-specific primary CD8 T-cell lines from freshly isolated or cryostored PBMC. Briefly, we incubated 4–8 × 10^{6} autologous B-lymphoblastoid cell line (BLCL) with the rhCMV-specific MOE peptide at 20–25 μM concentration for 90 min at 37°C, 5% CO₂. Next, we irradiated the cells with 9000rads and washed them three times to remove any unbound peptide. PBMC was then mixed with the peptide presenting B-LCL at a ratio of 1:1 in RPMI 1640 (GE Healthcare Life Sciences, Pittsburg, PA) medium supplemented with L-glutamine (GE), antibiotic/antimycotic (GE), and 15% fetal bovine serum (GE) (R15). Recombinant human interleukin-7 (Sigma-Aldrich, St. Louis, MO) was added at 10 ng/ml and the cell mixture was incubated 48 h at 37°C, 5% CO₂. Cells were then supplemented with R15 containing 100 units/ml recombinant human interleukin-2 (R&D Systems, Minneapolis, MN) (R15-100). Every 2–3 days, the media was refreshed with R15-100. We re-stimulated the
growing cell line with peptide-pulsed B-LCL weekly at a ratio of 1:1. These cells were used to determine MHC-I allele restriction in ICS assays between 4 and 6 weeks after the first stimulation.

Rhesus Macaque MHC Class I Molecule Transduced Cell Lines
Mamu-A1*002:01, or Mamu-B*001:01:02 expressing 721.221 cell lines were generated as described by (22). MHC class I-transduced 721.221 cells were maintained in R10 medium containing 0.5 mg/ml G418. The GenBank accession numbers for rhesus macaque MHC class I sequences are: Mamu-A1*002:01 (LN899621), Mamu-A1*008:01:01 (LN899628), and Mamu-B*001:01:02 (LM608018).

Tetramer and Surface Phenotype Staining
We pre-incubated 1 × 10⁶–3 × 10⁶ cells with APC-conjugated VV9, or NP8 tetramer at 37°C, 5% CO₂ for one hour. (The tetramers were produced by the NIH Tetramer Core Facility at Emory University, Atlanta, GA.) Next, we added the antibodies recognizing the surface phenotype markers. We used antibodies from BD Biosciences: CCR7 (clone 150503, R&D Systems) FITC, CD3 (clone SP34-2) PE-CF594, CD8 (clone RPA-T8) BV711, CD20 (clone 2H7) Alexa700, CD28 (clone CD28.2), PE, CD45 (clone D058-1283) BV786; from BioLegend: NKG2a (clone Z199) PE-Cy7, and Near-Infrared Live/Dead Discriminator to exclude dead cells. After fixation of the cells with 2% PFA. We acquired the data on a special-order BD LSR II (BD Biosciences, San Jose, CA) equipped with a 50 mW 405 nm violet, a 100 mW 488 nm blue, and a 50 mW 640 nm red laser, 16 detectors, and FACSDiva software version 8.0.1. We collected up to 250,000 events in the lymphocyte gate defined by the forward and side scatter parameters. We analyzed the data using FlowJo version 10.4.2.

Intracellular Cytokine Staining (ICS)
We incubated up to 3 × 10⁶ cells with 2.5 μM peptides or peptide pools in the presence of costimulatory antibodies CD28 (clone L293), CD49d (clone 9F10), and CD107a (clone H4A3) PE antibody, 0.5 μg brefeldin-A and 0.1 mm monensin (BioLegend, San Diego, CA) equipped with a 50 mW 405 nm violet, a 100 mW 488 nm blue, and a 50 mW 640 nm red laser, 16 detectors, and FACSDiva software version 8.0.1. We collected up to 250,000 events in the lymphocyte gate defined by the forward and side scatter parameters. We analyzed the data using FlowJo version 10.4.2.

rhCMV Viral Load Quantitation
We isolated DNA from 200 μl fluid samples (plasma, or urine), or 5 × 10⁶ cells using the Qiamp Blood DNA Mini kit (Qiagen, Germantown, MD). We eluted the DNA in 50 μl DEPC water following an additional 5-min incubation at room temperature. We tested the samples for rhCMV by QPCR using the Express QPCR Supermix Universal Kit (ThermoFisher Scientific, Waltham, MA) on a LightCycler 96 (Roche, Indianapolis, IN). Cycling conditions were: 95°C for 5 min followed by 50 cycles of 95°C for 1 s, 60°C for 1 min, and 72°C for 1 s. We used primers targeting the rhCMV UL54 gene (23). The final concentration of each primer was 300 nm. Forward primer – 5′-GATGGGAC CGCTCAAGTTTC-3′, reverse primer – 5′-TGACGGTAG CGAGGAGAAC-3′.

The final concentration of probe – (6FAM) GGTC GATGGGTTTTAGCTACGA (BHQ1) was 100 nm. We determined the concentration of the rhCMV DNA in the samples by interpolating onto a standard curve created by tenfold serial dilutions of a fragment of the rhCMV UL54 gene.

In Situ Tetramer Staining Combined with Immunohistochemistry
Fresh tissue sectioning, MHC Class I in situ tetramer staining, immunohistochemistry, and confocal imaging was performed as previously described (24, 25). Briefly, we incubated the tissue sections with 0.5 μg/ml FITC-labeled MHC-Class I tetramers overnight at 4°C. For negative control, we used FITC-labeled Mamu-A*001:01 tetramers loaded with an irrelevant, FLP (FLPSDFPSV) peptide from the hepatitis B viral core protein. For secondary staining, we incubated the sections with 0.5 μg/ml rabbit-anti-FITC Abs (AbDSerotec, Raleigh, NC), 0.19 μg/ml mouse-anti-human CD20 (clone L26) (Novocastra, Leica Microsystems, Buffalo Grove, IL), and 2 μg/ml rat-anti-human CD3 (clone CD3-12) (ThermoFisher) overnight at 4°C. For tertiary staining, we incubated the sections with 0.3 μg/ml Cy3-conjugated goat-anti-rabbit Abs, 0.75 μg/ml Alexa 488-conjugated goat-anti-rat Abs, and 0.75 μg/ml Cy5-conjugated goat anti-mouse Abs overnight at 4°C. We obtained all three tertiary antibodies from Jackson ImmunoResearch Laboratories (West Grove, PA). We counterstained selected sections with 1 μg/ml DAPI nuclear stain during the tertiary staining. We collected montage images of multiple 512 × 512-pixel z-series using a Leica confocal microscope (step size of 3 μm; starting 5–10 μm from the tissue surface to at least 45 μm deep into each section). B-cell follicles were identified morphologically as clusters of brightly stained, closely aggregated CD20+ cells, and delineated with Leica software. We used the same software to create montages of multiple projected confocal serial z-scans. Tops and bottoms of tetramer+ cells at a single Z-scan were distinguished from non-specific staining by stepping up and down through the adjacent z-scans.

Statistics
For statistical analysis we employed Fisher’s exact, or Students’ t test as indicated, using Microsoft Excel software version 16.16.1 for Macintosh.
RESULTS

Definition of rhCMV IE-1 and pp65-2 CD8 T Cell Epitopes

Initially we screened blood samples from 38 rhesus macaques to find responses elicited by 15-mers of the IE-1 and pp65-2 viral proteins using IFN-γ elispot. Among the 13 MHC-I alleles represented in this 38-animal cohort, 3 were shared by at least 2, and 10 were shared by at least eight animals as detailed in Supplementary Table 1. Mamu-A1*001:01, A1*002:01, A1*008:01, B*001, B*008, B*017 among others were included in the common alleles. Two responses, shared by multiple animals, were confirmed to be CD8 T-cell responses in subsequent ICS analysis. After deconvoluting the pools, and testing progressively shorter peptides we identified the following minimal optimal epitopes: VY9 - VTTLGMALY (aa291-299) of IE-1 (Fig. 1A), and NP8 - NPTDRPPI (aa96-103) of the viral protein pp65-2 (Fig. 1B). To assure that VY9 and NP8 are truly the minimal optimal epitopes, we performed ICS assays with serial tenfold dilutions of the relevant 9-mer and 8-mer peptides. As the one to two logarithmic lower EC50s of VY9 versus TY8, or NP8 versus HP9 and PP7 show we defined the correct minimal optimal epitopes (Supplementary Fig. 1). Comparative analysis of the MHC-I haplotype of the responding animals enabled us to narrow the presenting alleles to Mamu-A1*002:01 for the VY9, and Mamu-A1*008:01 for NP8. To support our hypothesis, we screened PBMC samples from additional animals with the minimal optimal epitopes, and found 14 out of 28 Mamu-A1*002:01 positive, and 0 out of 10 Mamu-A1*002:01 negative animals harboring VY9-specific cells. NP8 elicited response in 24 samples out of 28 Mamu-A1*008:01 positive, and 0 of 15 Mamu-A1*008:01 negative animals. Since these samples included PBMC from both healthy and SIV-infected animals at the chronic phase of the disease, we analyzed if there was a difference between the frequency, or the magnitude of elispot responses of the two groups. As shown in Supplementary Table 2, the magnitude of average IFN-γ response elicited by either the VY9 or NP8 epitope was slightly elevated in the SIV-infected animals, but the differences did not reach significant levels.

To confirm the identity of the restricting MHC-I alleles, we performed ICS assay using VY9 or NP8-specific CD8 T-cell lines and selected Mamu-MHC-I allele expressing transfecters (Fig. 1CD).

We had VY9, and NP8 tetrakers produced, and stained PBMC samples to quantitate cell populations that recognize these epitopes. We discovered the VY9 tetramer to be bound to both CD3+CD8+ and CD3-CD8- cells in approximately 75% of the Mamu-A1*002:01 positive animals (Fig. 2A,B; frequency of tetramer positive CD3+CD8+ cells: median = 3.67%, range 1.41–21.7%, n = 18, frequency of tetramer positive CD3-CD8- cells: median = 1.38%, range 0.05–16.55%, n = 24). The VY9 tetramer did not bind to cells obtained from Mamu-A1*002:01 negative animals (Supplementary Fig. 2). Previous results demonstrated that certain Killer Inhibitory Receptors bind to Mamu-MHC-I molecules containing the Bw6 motif (26). Therefore, the staining pattern we experienced with the VY9 tetramer was not surprising, in fact it was anticipated. Data showing that the VY9CD3-CD8+ cells were PD-1+, expressed the NK cell marker NKG2A/C (Fig. 2C), and could be double stained with the Mamu-A1*002:01 MHC-I restricted SIVmac239 gag LY9 tetramer further confirmed that the VY9CD3-CD8+ were in fact NK cells (Fig. 2D, E). The NP8 tetramer bound only to T cells in the Mamu-A1*008:01 positive animals (data not shown).

Tissue Distribution, Phenotype and Functional Capability of rhCMV-Specific T Cells

Analyzing blood samples from a cohort of healthy animals showed, that VY9-specific cells were present at higher frequency (average ± SD = 1.41 ± 1.03% of CD8+ T cells, range = 0.068–3.46%, n = 8) than NP8-specific cells in circulation (average ± SD = 0.35 ± 0.54% of CD8+ T cells, range = 0.026–1.92%, n = 11; Fig. 3A). The two populations differed significantly in their cellular composition as well: while the overwhelming majority of the VY9-specific cells were of CCR7-CD28- effector memory phenotype (TEM2) (average ± SD = 87.99 ± 12.97% of tetramer positive population, range = 60.4–99.3%, n = 8), the NP8-specific population contained fewer TEM2 cells (average ± SD = 45.61 ± 28.06% of tetramer positive population, range = 8.22–95.7%, n = 11; Fig. 3B). This pp65-2 epitope-specific cell population frequently included a large portion of CD28+CCR7+ TEM1 phenotype (9 of 11 samples contained >30% TEM1 within the tetramer positive population, average ± SD = 47.24 ± 23.94% range = 4.17–80.8%), and a minor CD28+CCR7+ central memory population (average ± SD = 7.03 ± 5.11% range = 0–17.8%). These significant differences suggest that peptides derived from the early IE-1 protein are presented on many more target cells, than peptides from the late pp65-2 protein.

Cytomegaloviruses have broad cell tropisms. Epithelial cells, fibroblasts, myeloid cells, hematopoietic progenitors, endothelial cells, and B cells are susceptible targets (27-30). Consequently, the presence of rhCMV-specific cells throughout the body is highly predictable. However, immune compartments of different anatomical locations must conform with different physiological demands. The cells’ functional adaptation can be traced by phenotypic and functional markers. Therefore, we investigated the distribution of VY9- or NP8-specific CD8+ T cells in a variety of tissues of four healthy adult animals. Multiple rhCMV DNA positive tissue samples of the animals provided evidence that all four of them were harboring the virus (Supplementary Table 3). In addition to the blood, we included samples from the bone marrow, the axillary, inguinal, and mesenteric lymph nodes and the spleen. CMV has been implicated in vascular diseases (31–33), thus we looked at the aorta and the inferior vena cava. Since the virus is shed in the urine and saliva, we included samples from the kidney, urinary bladder, lung and salivary gland. We investigated the frequency, the differentiation state, and the functional capability of these cells...
As anticipated, CMV-specific cells were relatively rare (range = 0.0035–0.31% of CD3+CD8+ T cells) in the lymph nodes, and just a small fraction of them reached the terminally differentiated TEM2 state (CCR7−CD28−) (range = 4.08–31.8% of tetramer positive T cells). In accordance to their surface phenotype, the majority of them were capable of producing IFN-γ and TNF-α, but frequently lacked cytotoxic capability (measured by CD107a expression) (Fig. 4C). Unexpectedly however, rhCMV-specific T cells were enriched in the spleen of three of the four animals (VY9 in r08026 and r07034, NP8 in r13053; range = 0.73–2.72% of CD3+CD8+ T cells). These cells were overwhelmingly of the TEM2 phenotype (>80% of the tetramer positive cells). Similar enrichment and phenotype were detected at the tertiary immune locations (kidney, urinary bladder, blood vessels, lung, salivary gland, and bone marrow) of the same animals (Fig. 4B). In r08026, VY9-specific CD8+ T cells represented unusually high frequencies in the aorta, kidney, and urinary bladder (Fig. 4A, Supplementary Fig. 3). r14073 appeared to be an outlier, since we found no enrichment of the
Fig 2. Mamu-A1*002:01 VY9 tetramer binds to both T and NK cells. (A) VY9 staining of live, singlet CD20− cells. (B) VY9 staining pattern of the cells gated for CD8 expression. (C) PD-1 and NKG2A/C expression by VY9+CD3−CD8+ (blue), and VY9+CD3+CD8+ cells (red). (D,E) GY9 and VY9 tetramer co-staining of a PBMC sample from an SIVmac239-infected animal. Live, singlet CD20−CD8+ cells were included in the gating. (E) CD3 antigen expression profile of the tetramer gated populations from panel (D). Red histogram shows the GY9-VY9 double positive population. The blue histogram shows the VY9 single positive population. The orange histogram shows the GY9 single positive population.

Fig 3. Frequency and TEM2 phenotype expression of the Mamu-A1*002:01 VY9 and Mamu-A1*008:01 NP8 cell populations in the peripheral blood. (A) VY9 (n = 8) and NP8 (n = 11) tetramer frequency in the peripheral blood of healthy rhesus macaques. Live, singlet, CD3+CD8+ tetramer+ lymphocytes were included in the gating strategy. P = 0.0045 (statistical analysis was performed using Student’s t test, unpaired, one-tailed distribution). (B) TEM2 cell frequency within the tetramer population of the blood. P = 0.0005 (statistical analysis was performed using Student’s t test, unpaired, one-tailed distribution). Live, singlet, CD3+CD8+ tetramer+ CCR7−CD28+ lymphocytes were included in the gating strategy.
Similar differentiation states across various tissues raises the possibility of similar functional phenotypes. Therefore, we analyzed the functional potential of VY9- or NP8-specific T cells in tissues where the number of isolated lymphocytes allowed us to perform intracellular cytokine staining (blood, spleen, lymph nodes, bone marrow, and lung). Interestingly, we could not detect IFN-γ, TNF-α, or CD107α response to VY9 peptide stimulation in r08026, in spite of the presence of tetramer positive cells well above the detection limit (e.g., 1.56% of CD3+CD8+ cells in the spleen) (Supplementary Fig. 4). We later found one more among the seven healthy animals tested, where VY9-specific T cells did not secrete IFN-γ, TNF-α, or expressed CD107α as a result of stimulation. (We did not observe similar lack of function for NP8-specific cells.) Data from the remaining three animals showed highly variable functional capability. This was true for different samples within the same animal, and for the same type of samples between different animals. In general, however, a higher proportion of the cells in the blood and spleen were cytotoxic, than the ones in the lung, or bone marrow (Fig. 4C).

**In Situ Staining with rhCMV-Specific Tetramers**

Defining the localization of virus-specific T cells in different histological areas of the various organs provides critical clues about the control of pathogen proliferation within the host. For example, data from parallel in situ tetramer staining and SIV RNA hybridization provided evidence that the B-cell areas of secondary lymphoid tissues serve as SIV virus reservoirs (34, 35). Therefore, we determined whether our tetramers could be used for in situ tetramer staining assays. We tested multiple spleen samples from the Mamu-A1*002:01 positive r07034 and the Mamu-A1*008:01 positive r13053 and found excellent staining with both tetramers. The VY9+ cells were limited to the extrafollicular area, frequently in close contact with B cells (Fig. 5A,B). NP8-specific cells translocated to the B-cell area, although the distribution was not uniform even within the same sample slide. Some follicles harbored abundant number of NP8-specific cells (Fig. 6A,B), others were devoid of them (Fig. 6C,D). We did not detect significant background staining (Fig. 6E,F).

**DISCUSSION**

Here, we report the development of new reagents and a staining strategy to study rhCMV-specific CD8 T-cell responses. The Mamu-A1*002:01 allele frequency is close to 20% and the Mamu-A1*008:01 frequency is approximately 25% of the rhesus macaques in the National Primate Research Centers. Therefore, the tetramers against these two epitopes combined with intracellular cytokine assays allow the research community to study rhCMV-specific responses in more detail in a large portion of the colony animals. This is especially important during chronic viral infection when virus-specific T cells can become exhausted and cease cytokine secretion.
Tetramer staining offers the possibility to detect and quantify these exhausted populations.

We also provided tools to isolate viable rhCMV-specific CD8 T cells by flow cytometry sorting with these new reagents. It is important to emphasize that one of the two tetramers binds to both NK and T cells. While NK cells and CD8 T cells can be distinguished using several surface markers, we found that gating based on the expression of NKG2A/C yielded the most reliable results. NK cells do not express CD3, but rhCMV-specific CD8 T cells frequently downregulated their CD3 level to an extent that separation of the negative and dim population was difficult. For the same reason, the selection of an anti-CD3 antibody conjugated to a bright fluorochrome was essential in the staining panel. Another highly expressed characteristic NK cell marker is CD16 (37). However, this antigen is shed very easily from the cells after a freeze–thaw cycle; therefore, its application as a distinguishing marker on cryostored samples is not appropriate (38, 39). NKG2A/C is expressed at high level on NK cells, and can be expressed at low level on CD8 T cells. Separation of NK cells and CD8 T cells in rhesus macaque samples based on the expression of NKG2A/C is unequivocal.

In the present study, we found that the frequency and memory phenotype of VY9 and NP8 epitope-specific T cells is significantly different in the blood, providing evidence that the individual CMV epitopes are at different stages of a progressive differentiation. These data correspond well both with murine data (40), showing different memory CD8 T-cell phenotypes during chronic MCMV infection, and with the concept of continuous, antigen-driven differentiation of CMV-specific T cells (41). Additionally, our results correspond to the data from other laboratories, indicating that the magnitude of CMV-specific CD8 T-cell responses tend to be higher against viral proteins that are expressed early in the viral life cycle than the ones expressed later. For example, Sylwester et al. demonstrated that hCMV-specific CD8 T cells against immediate early genes are close to two times more frequent than the ones against late genes (42). Khan et al. provided data showing that the HLA-A*0201 restricted IE-1 Vl9 population is greater than the pp65 Vl9 in those adult individuals that possess both responses (43), and Gibson et al. found the same in acutely infected young children (44).

We demonstrated that in addition to blood, lymph nodes, and spleen, a sufficient number of lymphocytes can be isolated from lung, and bone marrow samples to perform both surface phenotype and functional analysis of rhCMV-specific CD8 T cells. It has been established that the functional repertoire of CD8 T cells is influenced by the particular microenvironment where they reside (45). Our data provide an initial glimpse into this compartmentalization. We detected a relative absence of CD107a positive rhCMV-specific cells in the lung and bone marrow, as opposed to the blood and spleen of the same host, where the majority of rhCMV-specific cells possessed this marker. Our results echo the previous findings of Chan et al., showing that the functional capability of circulating rhCMV-specific CD8 T-cell populations is heterogeneous within individual animals and between different epitopes, with a significant fraction possessing cytolytic capacity (46).

We indicated the value of these tetramers in defining the tissue localization of rhCMV-specific cells. Our in situ
tetramer staining images provided evidence on the close contact between the VY9-specific T cells and CD20+ B cells within the extrafollicular area. Previously published in vitro data suggested that B cells can be infected with CMV, but the virus is unable to complete its life cycle (47). Whether the close contact between the VY9-specific T cells and B cells reflects this abortive infection or B cells express this epitope through other mechanism needs to be determined in future studies. The in situ tetramer staining with NP8 tetramer demonstrated that these cells are not evenly distributed within the

Fig 6. Localization of rhCMV NP8-specific cells in the spleen. (A,C) Montages of multiple confocal projected serial z-scans of a sample of animal r13053 (Mamu-A1*008:01+) stained with Mamu-A1*008:01 NP8 tetramer. (B,D) Enlargement from (A) and (C), respectively, showing the range of distribution patterns of rhCMV NP8-specific CTL within follicular (F) and extrafollicular areas (EF) (a single confocal z-scan). (B) Demonstrates a follicle rich with rhCMV NP8-specific CTL that are uniformly distributed within the B-cell follicle. (D) Demonstrates a B-cell follicle with few rhCMV NP8-specific CTL located near the edge of the follicle. rhCMV NP8-specific CTL red, CD3+ cells blue, and CD20+ B cells: green color. Tetramer+ cells are indicated by white arrowheads. Panel (E) (negative control) shows a montage of multiple confocal projected serial z-scans from a spleen tissue section from animal r07034 (Mamu-A1*002:01+) rhesus macaques stained with Mamu-A1*008:01 NP8 tetramers. (F) Enlargement from (E) and shows a single confocal z-scans showing that no rhCMV NP8-specific CTL were detected in the tissue. Confocal images were collected using a 20x objective. Scale bars = 100 μm.
numerous follicular areas of the same secondary lymphoid tissue. This technique may provide the tool to understand rhCMV latency in the presence of strong cellular immune responses.

As a summary, here we present for the first time the definition of two new, classical MHC-I restricted rhCMV-specific T-cell epitopes. The reagents developed with these epitopes will advance the efforts aiming to understand CMV-specific cellular immune responses in one of the most important large animal models of human diseases.

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AUTHOR CONTRIBUTIONS

Nicholas Pomplun: Data curation. Logan Vosler: Data curation. Kimberly Weisgrau: Data curation. Jessica Furlott: Data curation. Andrea Weiler: Data curation. Hadi Abdeelal: Data curation. David Evans: Formal analysis; resources. David Watkins: Conceptualization; resources. Tetsuro Matano: Resources. Pamela Skinner: Formal analysis; resources; supervision. Thomas Friedrich: Formal analysis; methodology. Eva Rakasz: Conceptualization; formal analysis; project administration; supervision; writing-original draft; writing-review and editing.

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