Cytomegalovirus pp65 limits dissemination but is dispensable for persistence

Daniel Malouli,1 Scott G. Hansen,1 Ernesto S. Nakayasu,2 Emily E. Marshall,1 Colette M. Hughes,1 Abigail B. Ventura,1 Roxanne M. Gilbride,1 Matthew S. Lewis,1 Guangwu Xu,1 Craig Kreklywich,1 Nathan Whizin,1 Miranda Fischer,3 Alfred W. Legasse,3 Kasinath Viswanathan,1 Don Siess,3 David G. Camp II,2 Michael K. Axthelm,3 Christoph Kahl,3 Victor R. DeFilippis,1 Richard D. Smith,2 Daniel N. Streblow,1 Louis J. Picker,1,3 and Klaus Früh1,3

1Vaccine and Gene Therapy Institute, Oregon Health and Science University, Beaverton, Oregon, USA.
2Biological Sciences Division, Pacific Northwest National Laboratory, Richland, Washington, USA.
3Oregon National Primate Research Center (ONPRC), Oregon Health and Science University, Beaverton, Oregon, USA.

The most abundantly produced virion protein in human cytomegalovirus (HCMV) is the immunodominant phosphoprotein 65 (pp65), which is frequently included in CMV vaccines. Although it is nonessential for in vitro CMV growth, pp65 displays immunomodulatory functions that support a potential role in primary and/or persistent infection. To determine the contribution of pp65 to CMV infection and immunity, we generated a rhesus CMV lacking both pp65 orthologs (RhCMVΔpp65ab). While deletion of pp65ab slightly reduced growth in vitro and increased defective particle formation, the protein composition of secreted virions was largely unchanged. Interestingly, pp65 was not required for primary and persistent infection in animals. Immune responses induced by RhCMVΔpp65ab did not prevent reinfection with rhesus CMV; however, reinfection with RhCMVΔUS2-11, which lacks viral-encoded MHC-I antigen presentation inhibitors, was prevented. Unexpectedly, induction of pp65b-specific T cells alone did not protect against RhCMVΔUS2-11 challenge, suggesting that T cells targeting multiple CMV antigens are required for protection. However, pp65-specific immunity was crucial for controlling viral dissemination during primary infection, as indicated by the marked increase of RhCMVΔpp65ab genome copies in CMV-naive, but not CMV-immune, animals.

Authorship note: Daniel Malouli and Scott G. Hansen contributed equally to this work.

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el to study CMV and CMV vaccines (32–34). Since host restriction resulted in coevolution of CMVs with their respective hosts, infection of rhesus macaques (RMs) with rhesus CMV (RhCMV) represents an animal model that closely resembles infection of humans with HCMV (35). We therefore used this model to study the role of RhCMV pp65 in infection and immunity. RhCMV encodes 2 ORFs, Rh111 and Rh112, with comparable homology to HCMV pp65 (pp65a ~34%, pp65b ~40%) and 40% identity to each other (36, 37). The 2 proteins combined comprise approximately 11% of the entire viral proteome in RhCMV virions (38), which is similar to HCMV, in which the single pp65 protein makes up 15% of the virion proteins (18). To examine the function of pp65 in vitro and in vivo, we deleted both pp65 homologs from the genome of RhCMV. We characterized the impact of pp65 deletion on viral growth in vitro and on the composition of the virion proteome. We then determined the role of pp65 for the ability of RhCMV to establish primary or secondary persistent infection in RhCMV+ or RhCMV– animals, respectively. By challenging with recombinant RhCMV lacking the immunoevasins US2, 3, 6, and 11, a virus incapable of superinfecting, we further evaluated whether pp65-specific T cells are required for the protective effect of preexisting CMV infection or sufficient to recapitulate T cell–mediated protection induced by natural infection. Our observations demonstrate a unique physiologic role for pp65 in CMV biology and, moreover, have implications for the use of pp65 as a subunit vaccine.

**Results**

**RhCMVΔpp65ab shows delayed growth kinetics at low MOI.** To study the function of pp65 in RMs, we deleted the pp65a- and pp65b-encoding genes Rh111 and Rh112 in the RhCMV strain 68-1–derived BAC (39) to generate Δpp65ab. Upon reconstitution of recombinant virus in telomerized rhesus fibroblasts (TRFs), we verified that genes Rh111 and Rh112 were absent, whereas the neighboring genes Rh110 (UL82 [pp71] homolog) and Rh114 (UL84 homolog) were still expressed (Figure 1A).

To determine whether pp65 deletion affected in vitro growth properties of RhCMV, we compared the growth kinetics of Δpp65ab with those of BAC-derived RhCMV 68-1 (herein referred to as WT control). TRFs were infected with Δpp65ab or WT either at a high MOI of 3 to generate a single-step growth curve or at a low MOI of 0.01 to measure multistep growth. Supernatants collected at high MOI contained similar titers of the 2 viruses, with a peak titer reached on and after 4 days postinfection (dpi) (Figure 1B). However, when multiple rounds of infection were measured, Δpp65ab displayed a modest, but significant, delay in viral growth, ultimately reaching the same peak titer as WT (Figure 1C).

**Characterization of Δpp65ab virions.** Since previous reports suggested that pp65 in HCMV affected viral assembly and thus the incorporation of other viral proteins (31), we studied the structure and protein composition of Δpp65ab virions. Using mass spectrometry, we demonstrated recently a remarkable similarity between the predominant viral proteins found in both RhCMV and HCMV, with respect to protein ratios and protein abundance (38). To similarly determine the proteome of Δpp65ab virions, we concentrated viral particles from the supernatant of infected TRFs over a discontinuous Nycodenz gradient (see Methods). Compared with WT, we observed an increased appearance of particles that sedimented with higher density in virion preparation of Δpp65ab (Figure 2A). Electron microscopy of this high-density band revealed abormal structures consistent with that of WT, suggesting that pp65 is essential for normal virion morphology.
with capsidless (defective) viral particles. However, the lower density virion band contained an essentially pure preparation of particles with the same general structure as WT RhCMV, including an icosahedral capsid containing the viral DNA as the core of the virion surrounded by a tegument layer and enveloped by a lipid membrane (Figure 2B). NuPAGE and Western blot analysis of gradient-purified WT and Δpp65ab virions demonstrated the absence of pp65a and pp65b in the deletion mutant (Figure 2, C and D). Comparison of the dimensions of the WT versus Δpp65ab virions revealed an overall reduced diameter of Δpp65ab virions (173.4 nm) compared with that of WT virions (222.5 nm) (Figure 2E). This reduction in particle size was primarily due to a significant reduction of the viral tegument layer (38.4 nm [Δpp65ab] compared with 61.2 nm [WT]), consistent with the fact that pp65a and pp65b constitute a major portion of the viral tegument in WT (approximately 24.4%; ref. 38). In addition, the capsid appeared to be diminished in size, although to a lesser degree (89.7 nm [Δpp65ab] compared with 100.2 nm [WT]). Thus, both phenotypically normal, but smaller, virions and defective particles were recovered from the supernatant of cells infected with Δpp65ab.

To further characterize the proteome of the virions contained in the upper band by mass spectrometry, the recovered material was digested with trypsin and analyzed by 1D LC-MS/MS. The resulting mass spectra were initially searched against stop-to-stop translated sequences of the RhCMV 68-1 BAC genome for ORFs ≥30 amino acids. Since all proteins identified by this method corresponded to proteins contained in our recently published reannotation of the RhCMV 68-1 BAC genome (38), we also used a protein library based on the revised annotation for further analysis of protein abundance. The virion preparation
### Table 1
Comparison of viral proteins contained in WT and Δpp65ab virions

| ORF RhCMV | ORF HCMV | Description | Group | Mol% in virion (RhCMV WT)
|-----------|-----------|-------------|-------|---------------------------|
| Rh112     | UL83      | Tegument protein pp65b | A     | 7.23
| Rh118     | UL86      | Major capsid protein (MCP) | C     | 7.22
| Rh117     | UL85      | Minor capsid protein (mCP) | C     | 6.63
| Rh129     | UL94      | Tegument protein | A     | 4.88
| Rh102     | UL73      | Envelope glycoprotein N | B     | 4.83
| Rh44      | UL26      | Tegument protein | A     | 4.16
| Rh59      | UL35      | Tegument protein | A     | 3.81
| Rh111     | UL83      | Tegument protein pp65a | A     | 3.76
| Rh138     | UL100     | Envelope glycoprotein M | B     | 3.70
| Rh75      | UL46      | Triplex capsid protein, subunit 1 | C     | 3.45
| Rh104     | UL75      | Envelope glycoprotein H | B     | 3.17
| Rh137     | UL99      | Myristoylated tegument protein | A     | 2.97
| Rh43      | UL25      | Tegument protein | A     | 2.86
| Rh100.1   | UL71      | Tegument protein | A     | 2.75
| Rh125     | US28      | G protein–coupled receptor homolog (vGPCR2) | B     | 2.75
| Rh110     | UL82      | Tegument protein pp71 | A     | 2.73
| Rh230     | TRS1      | Tegument protein | D     | 2.43
| Rh88      | UL55      | Envelope glycoprotein B | B     | 2.38
| Rh79.1    | UL48A     | Small capsid protein | C     | 2.34
| Rh203     | US22      | Tegument protein | A     | 2.17
| Rh72      | UL45      | Ribonucleotide reductase subunit 1 (R1) | D     | 2.10
| Rh148     | UL116     | Tegument protein | E     | 1.66
| Rh76      | UL47      | Tegument protein | A     | 1.51
| Rh78      | UL48      | Large tegument protein | A     | 1.42
| Rh140     | UL103     | Tegument protein | A     | 1.34
| Rh123     | UL88      | Potential Tegument protein | E     | 1.32
| Rh106     | UL77      | Virion-packaging protein | C     | 1.21
| Rh147     | UL115     | Envelope glycoprotein L | B     | 1.03
| Rh164     | UL141     | Membrane glycoprotein | B     | 0.92
| Rh132     | UL97      | Phosphotransferase | D     | 0.87
| Rh211     | US26      | Envelope glycoprotein | B     | 0.85
| Rh160     | UL132     | Envelope glycoprotein | B     | 0.81
| Rh56      | UL33      | GPCR homolog, envelope glycoprotein | B     | 0.78
| Rh152/Rh151 | UL119/UL118 | Membrane glycoprotein, viral Fc-γ receptor | B     | 0.70
| Rh128     | UL93      | Capsid-associated protein | C     | 0.64
| Rh141     | UL104     | Capsid portal protein | C     | 0.47
| Rh216     | US28      | GPCR homolog (vGPCR3B) | B     | 0.36
| Rh131     | UL96      | Tegument protein | A     | 0.35
| Rh17      | RL11 family | E     | 0.32
| Rh214     | US28      | GPCR homolog (vGPCR1) | B     | 0.28
| Rh42      | UL24      | Tegument protein | A     | 0.28
| Rh173     | RL11 family | E     | 0.27
| Rh218     | US28      | GPCR homolog (vGPCR4) | B     | 0.27
| Rh164.1   | E         | 0.23
| Rh103     | UL74      | Envelope glycoprotein O | B     | 0.20
| Rh165     | E         | 0.18
| Rh131     | RL13      | Membrane protein | B     | 0.14
| Rh109     | UL80      | Capsid maturation protease | C     | 0.10
| Rh70      | UL44      | DNA polymerase processivity factor | D     | 0.10
| Rh805     | RL11 family | E     | 0.09
| Rh156 (IE2) | UL122    | Immediate-early protein 2, pp96 | D     | 0.08
| Rh81      | UL50      | Nuclear egress membrane protein | B     | 0.04
| Rh83      | UL52      | Packaging protein | D     | 0.00
| Rh134     | UL98      | Deoxyribonuclease | D     | 0.00
| Rh114     | UL84      | D     | 0.00

*The identified proteins were separated into 5 different groups dependent on their subcellular localization or function: A, tegument; B, envelope and glycoproteins; C, capsid; D, transcription/replication machinery; and E, uncharacterized. *These data are based on WT proteomics results published by Malouli et al. (38). Asterisks indicate proteins that are not present in either the WT or Δpp65ab sample, so fold changes cannot be calculated.
was analyzed by LC-MS/MS upon elution from 0% to 100% acetonitrile gradient over 100 minutes, and 5 technical repeats were performed. 68.5 mol% of all identified proteins and 69.9 mol% of all identified peptides corresponded to viral proteins, whereas 31.5 mol% of the proteins and 30.1% of the peptides were derived from the host (Supplemental Figure 1B; supplemental material available online with this article; doi:10.1172/JCI67420DS1). This result is similar to that previously obtained for WT (64.8 mol% viral proteins, 63.4% viral peptides and 35.2 mol% host proteins, 36.6% host peptides), suggesting a similar level of sample purity. As expected, peptides corresponding to pp65a or pp65b were not detected in the Δpp65ab virion preparation (Table 1). However, a total of 50 different viral proteins could be identified for Δpp65ab, which is comparable to that for WT virions, for which 53 different viral proteins were identified. Every capsid protein found in WT virions was found in the deletion mutant, and besides the deleted pp65 proteins, this also holds true for the tegument proteins (Table 1). Similarly, all major glycoproteins were present in Δpp65ab in equal abundance compared to WT. In fact, most proteins that differed in their abundance between Δpp65ab virions compared with WT were low-abundance proteins, suggesting that these proteins might not be consistently part of the virions or that they were missed in our analysis due to low abundance. If an abundance threshold of 0.25 mol% is applied, 8 proteins differ between Δpp65ab and WT: Rh17 (RL11 family), Rh131 (UL96), Rh211 (US26), and Rh214 (US28) are decreased in the Δpp65ab mutant compared with the WT, whereas Rh05 (RL11 family), Rh13.1 (RL13), Rh173 (RL11 family), and Rh218 (US28) were increased in the Δpp65ab mutant compared with the WT (Figure 3). Of those, Rh211 is the only protein with a substantial presence with 0.85 mol% in WT virions that is completely absent in mutant virions. Rh211 is the homolog of HCMV US28, whose function is unknown. Thus, our proteomics analysis revealed that only 8 proteins with a higher

Figure 3
Δpp65ab establishes primary and secondary infections and protects against superinfection with ΔUS2-11. (i) Two RhCMV seronegative male RMs (filled circles, Rh22037; open circles, Rh23016) were infected s.c. with 10^7 PFUs of Δpp65ab at day 1. CD4+ (blue) and CD8+ (red) T cell responses were monitored in peripheral blood (PBMCs) by intracellular cytokine staining at the indicated days using overlapping peptides of pp65ab and IE1/2. (ii) On day 659, the 2 animals were inoculated s.c. with 10^7 PFUs of ΔUS2-11 (green dotted line), and the T cell response to SIVgag was measured in addition. Note the absence of a T cell response to SIVgag or pp65 and a lack of boosting of responses to IE1. (iii) On day 876, the 2 RMs were inoculated with 10^7 PFUs of WTgag (black dotted line), and the T cell response was monitored by intracellular cytokine staining. Note the appearance of de novo responses to SIVgag and pp65 and a boosting of the T cell response to IE1. (iv) On day 1,107, the 2 RMs were inoculated with 10^7 PFUs of Δpp65ab-rtn (blue dotted line). Using overlapping 15-mer peptides, a de novo response to SIVrtaf was detectable, indicating superinfection. Also note a boosting of the IE1 response but not of pp65- or SIVgag-specific responses. The corresponding T cell responses obtained from BAL fluid are shown in Supplemental Figure 2.
abundance than 0.25 mol% showed marked changes of more than 2-fold between the WT and the pp65ab deletion mutant, with most of these being low-abundance proteins.

In general, there was surprisingly little impact of pp65ab deletion on the presence of other proteins in the virions. There was no substantial difference among nonstructural proteins (transcription/replication machinery or uncharacterized category), whereas the quantities of most structural proteins (capsid, envelope, glycoproteins) were, in fact, slightly elevated in Δpp65ab compared with those in WT (Table 2). Moreover, we did not observe a decrease in specific, non-pp65 tegument proteins, but rather, we saw a decrease in the abundance of all non-pp65 tegument proteins in Δpp65ab virions. This is in contrast to a previous report for HCMV, describing selective lack of specific tegument proteins in pp65-deleted virions (31). Thus, it seems that RhCMV virions assembled normally but with an overall reduced tegument. Indeed, when virion protein abundance is adjusted for the absence of pp65ab by normalizing to a total of 89% (11% of the WT virion is made up by pp65a and pp65b combined), protein quantities are very similar to those of WT (Tables 1 and 2). Despite the lack of major tegument proteins that normally represent 11% of the viral particle mass, there was little change in virion composition.

In contrast to the limited impact of pp65ab deletion on virion proteins, a number of host proteins were substantially different between WT and Δpp65ab (Supplemental Figure 1). 279 host proteins were identified in WT, whereas 240 host proteins were identified in Δpp65ab. Only 172 host proteins were identified in both viral samples, with the remaining proteins being unique to each sample. The role of host cell proteins in CMV virions is unknown, but it seems likely that these proteins reflect the source or host cell membrane used for envelopment. The differential presence of host cell proteins could thus indicate that envelopment of pp65ab-deleted viruses differs somewhat from that of WT virus. This would be consistent with the increased production of defective particles described above. The differential incorporation of host cell proteins likely reflects changes in viral assembly pathways but is less likely of consequence for virion function.

Table 2
Relative abundance of the 5 functionally different groups of viral proteins in RhCMV WT and Δpp65ab

<table>
<thead>
<tr>
<th>Protein Group</th>
<th>RhCMV 68-1</th>
<th>SD</th>
<th>RhCMVΔpp65ab</th>
<th>SD</th>
<th>P-value compared to 68-1</th>
<th>RhCMVΔpp65ab adjusted</th>
<th>SD</th>
<th>P-value compared to 68-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tegument</td>
<td>45.09</td>
<td>2.26</td>
<td>37.03</td>
<td>1.58</td>
<td>0.0005</td>
<td>32.95</td>
<td>1.40</td>
<td>0.00004</td>
</tr>
<tr>
<td>Envelope and glycoproteins</td>
<td>22.38</td>
<td>1.30</td>
<td>26.00</td>
<td>2.37</td>
<td>0.03</td>
<td>23.14</td>
<td>2.11</td>
<td>0.30</td>
</tr>
<tr>
<td>Capsid</td>
<td>22.03</td>
<td>1.58</td>
<td>26.46</td>
<td>1.07</td>
<td>0.001</td>
<td>23.55</td>
<td>0.95</td>
<td>0.07</td>
</tr>
<tr>
<td>Transcription/replication machinery</td>
<td>5.58</td>
<td>0.42</td>
<td>5.24</td>
<td>0.84</td>
<td>0.27</td>
<td>4.67</td>
<td>0.75</td>
<td>0.05</td>
</tr>
<tr>
<td>Uncharacterized</td>
<td>4.92</td>
<td>1.14</td>
<td>5.28</td>
<td>0.59</td>
<td>0.28</td>
<td>4.70</td>
<td>0.52</td>
<td>0.36</td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
<td></td>
<td>100.00</td>
<td></td>
<td>89.00</td>
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</table>

Figure 4
RhCMVΔpp65ab is persistently secreted from infected animals. (A) The time line depicts the time points of inoculation with different RhCMV constructs and the days when cocultures were started from urine. Time points marked with asterisks indicate additional days in which cocultures were positive for Δpp65ab, but the data are not shown. PID, postinoculation day. (B) Immunoblot for the indicated antigens in lysates from representative viral cocultures with urine collected on the indicated dpi. The presence of RhCMV-IE1, RhCMV-pp65b, SIVgag, and SIVtretanef in cell lysates was detected by immunoblot using antibodies specific for the respective antigens (IE, pp65) or for epitope tags fused to SIVgag or SIVtretanef. Note that, initially, secreted RhCMV expressed IE, but not pp65, whereas superinfection with WTgag and Δpp65tretanef is indicated by the appearance of pp65-containing virus expressing the respective antigens. As positive control (Con), coculture lysates from a RM inoculated with WTgag and WTtretanef is included.
research article

A. CD4+ and CD8+ lymphocyte responses over time after inoculation with MVA and US2-11gag.

B. Capture antigen: RhCMV-Δpp65 lysate.

C. Capture antigen: RhCMV-WT lysate.

D. Lysate: WT Δ65 and WT Δ65 with reduced exposure.
Infection of RMs with Δpp65ab. To determine whether pp65ab-deleted viruses would be infectious, we inoculated 2 seronegative male RMs with 10⁷ PFUs of Δpp65ab and monitored the CMV-specific T cell response using overlapping peptides to the RhCMV proteins IE1/2 and, as control, to pp65ab for about 22 months. We also monitored viral shedding by coculture of urine samples with rhesus fibroblasts. In previous experiments, we showed that infection of RhCMV-negative RMs results in the appearance of peak T cell responses within the first 2 weeks of infection, followed by a contraction and stabilization of the T cell response at a level that remains more or less constant for the duration of the life of the animal (40, 41). The maintenance of such a long-lived effector memory T cell response reflects the establishment of persistent infection. Similarly, both animals infected with Δpp65ab responded vigorously to IE1/2, with a peak CD⁴⁺ and CD⁸⁺ T cell response in PBMCs and bronchoalveolar lavage (BAL) fluid at 2 weeks, followed by a slow decline and stabilization of the response that lasted for the entire time (Figure 3, i, and Supplemental Figure 2A). Importantly, T cell responses to pp65ab were not observed, which is consistent with the IE1/2 responses being induced by the Δpp65ab-deleted virus. The stable T cell response to IE1/2 suggested that the pp65ab-deleted virus established persistent infection. Persistence was further confirmed by coculture of urine samples with TRFs, in which IE1 was detected in urine cocultures of Δpp65ab-infected animals but pp65 was not detected, confirming that there was no contamination with WT virus (Figure 4). Therefore, these data suggest that RhCMV is able to establish and maintain a persistent infection despite the absence of pp65ab.

Although pp65 is one of the major targets of the CMV-specific T cell response in both humans and monkeys (14–17, 37), the contribution of pp65-specific T cells to control of CMV replication is not known. Indeed, the experimental determination of the efficacy of RhCMV-specific T cell responses is complicated by the fact that RhCMV readily superinfects RhCMV⁺ RMs, overcoming preexisting T cell responses due to the presence of viral proteins that inhibit MHC-I antigen presentation (42). However, RhCMV lacking the genes encoding for homologs of the HCMV US2, 3, 6, and 11 immunoevasins is unable to superinfect CMV⁺ RMs but is capable of establishing persistent infection in CMV-naive animals or upon depletion of CD⁸⁺ T cells from CMV-immune animals (42). Thus, the ability to protect against superinfection with ΔUS2-11 RhCMV is a convenient surrogate marker for the quality of T cell responses, i.e., a T cell response that is as efficient as that induced by natural infection. To test whether the T cell responses induced by Δpp65ab to antigens other than pp65 would be sufficient to prevent superinfection with immunoevasins-deleted virus, we inoculated the 2 Δpp65ab-infected RMs with ΔVIHCEAUUS2-11 gag, a previously described recombinant virus that expresses the SIVgag as immunological marker. In addition to US2-11, this virus lacks the RhCMV-specific viral inhibitor of heavy chain expression (VIHCE), which is not required for superinfection (42). As observed for RMs naturally infected with RhCMV, ΔVIHCEAUUS2-11 gag was unable to superinfect Δpp65ab-infected animals, as evident from the absence of an immune response to SIVgag as well as a lack of a boosting response to IE or a de novo response to pp65 (Figure 3, ii, and Supplemental Figure 2B). In fact, T cell responses to IE1/2 remained stable for the entire duration of this experiment (200 days). Thus, the T cell responses generated by Δpp65ab were as efficacious as T cell responses induced by WT in protecting against immunoevasins-deleted virus challenge, indicating that a pp65-specific T cell response is not required for an effective anti-RhCMV immune response and that T cells specific for other codominant or subdominant antigens are sufficient for protection.

To determine whether animals infected with Δpp65ab are resistant to superinfection by WT RhCMV, we inoculated both RMs with WT-gag, a previously described virus that carries SIVgag inserted into the ORF Rh211 between hypothetical ORFs 213 and 214 (41). Upon inoculation of 10⁷ PFUS WT-gag, both animals displayed clear signs of superinfection, as evident by the development of de novo responses to SIVgag and pp65ab and by boosting of the preexisting T cell response to IE1/2 (Figure 3, iii, and Supplemental Figure 2C). Moreover, cocultures of urine samples from these animals contained SIVgag-expressing virus (Figure 4). These data thus demonstrate that the immune responses induced by Δpp65ab, like those elicited by WT RhCMV, are unable to protect against superinfection with WT RhCMV.

Given the role of HCMV pp65 as modulator of several immune response pathways (including protecting against IE-specific T cells [23] and NK cells [22]), it was possible that pp65 itself contributed to the ability of WT to overcome preexisting immune responses. In fact, our previous finding that evasion of T cell responses plays a central role in overcoming preexisting immune responses does not rule out that evasion of other immune response components, e.g., B cells and NK cells, might also contribute to superinfection (42). To examine whether RhCMV lacking pp65ab would be able to superinfect CMV-positive animals, we inserted an expression cassette for SIVtat and nef (a fusion protein of rev, int, tat, and nef; refs. 41, 43) into the RhCMV genome by replacing the pp65-encoding genes Rh111 and Rh112. After confirming pp65 deletion, in vitro growth properties, and expression of SIVtat and nef (data not shown), we inoculated the 2 RMs previously infected with Δpp65ab and WT-gag with Δpp65ab-retanef and monitored the immune response.
Table 3
Copy numbers of RhCMV WT-gag and Δpp65ab-retanef genomes in CMV+ RMs

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>14 dpi (Rh29036)</th>
<th>28 dpi (Rh29999)</th>
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<tr>
<td>RhCMV 68-1 Δpp65ab</td>
<td>RhCMV 68-1 Δpp65ab</td>
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<tr>
<td>Skin injection site (WT)</td>
<td>ND 18</td>
<td>ND 21</td>
</tr>
<tr>
<td>Skin injection site (Δpp65ab)</td>
<td>11 32</td>
<td>ND 23</td>
</tr>
<tr>
<td>Axillary lymph node (WT)</td>
<td>ND ND</td>
<td>ND ND</td>
</tr>
<tr>
<td>Axillary lymph node (Δpp65ab)</td>
<td>ND ND</td>
<td>ND ND</td>
</tr>
<tr>
<td>Iliosacral lymph node (WT)</td>
<td>ND ND</td>
<td>ND ND</td>
</tr>
<tr>
<td>Iliosacral lymph node (Δpp65ab)</td>
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<tr>
<td>Inguinal lymph node (WT)</td>
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<td>ND ND</td>
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<tr>
<td>Inguinal lymph node (Δpp65ab)</td>
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<td>ND ND</td>
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<td>ND ND</td>
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<td>Submandibular salivary gland (WT)</td>
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<tr>
<td>Submandibular salivary gland (Δpp65ab)</td>
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<td>ND ND</td>
</tr>
<tr>
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<td>ND 122</td>
<td>ND 12</td>
</tr>
<tr>
<td>Sublingual salivary gland (Δpp65ab)</td>
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<td>ND ND</td>
</tr>
<tr>
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<td>ND ND</td>
</tr>
<tr>
<td>Parotid salivary gland (Δpp65ab)</td>
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<td>ND ND</td>
</tr>
<tr>
<td>BAL pellet</td>
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<tr>
<td>Lung</td>
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<td>ND ND</td>
</tr>
<tr>
<td>Spleen</td>
<td>ND 5</td>
<td>ND ND</td>
</tr>
<tr>
<td>Liver</td>
<td>ND 2</td>
<td>ND ND</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>ND 12</td>
<td>ND ND</td>
</tr>
<tr>
<td>Urine</td>
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<td>ND ND</td>
</tr>
<tr>
<td>Brain</td>
<td>ND 9</td>
<td>ND ND</td>
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<tr>
<td>Spinal cord (lumbar)</td>
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<td>ND ND</td>
</tr>
<tr>
<td>Spinal cord (thoracic)</td>
<td>ND ND</td>
<td>ND ND</td>
</tr>
<tr>
<td>Spinal cord (cervical)</td>
<td>ND ND</td>
<td>ND ND</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>ND ND</td>
<td>ND ND</td>
</tr>
<tr>
<td>PBMC</td>
<td>ND ND</td>
<td>ND ND</td>
</tr>
<tr>
<td>Plasma</td>
<td>ND ND</td>
<td>ND ND</td>
</tr>
</tbody>
</table>

Genome copy numbers in tissue samples are given per 10^7 cell equivalents, whereas genome copies in urine and plasma are shown per ml. 

When tissues were harvested from both the left and right side, it is indicated in brackets whether the sample was derived from the side of WT or Δpp65ab infection. ND, not detected.

response to SIVretanef. As shown in Figure 3, iv (Supplemental Figure 2D), both animals showed clear signs of superinfection, as evident from the development of a de novo T cell response to SIVretanef and a boosting of the preexisting IE1/2 response. Note that the T cell responses to pp65ab and SIVgag were not boosted, confirming the lack of pp65ab and SIVgag. We thus conclude that pp65ab is dispensable for the establishment of both primary and secondary persistent infections.

Vaccine-induced pp65-specific T cells do not recapitulate the protective effect of T cells induced by natural infection. In the RM model, it was previously demonstrated that vaccination with subunit vaccines consisting of pp65b (with or without IE1) as T cell–inducing components and gB as neutralizing antibody-inducing component reduced RhCMV viremia and shedding (44–46). However, our data also suggest that T cell responses to antigens other than pp65 play an important role in the protective effect of RhCMV infection against AUS2-11 challenge. We were therefore wondering whether induction of a T cell response to pp65 alone would be sufficient to recapitulate the protective effect of preexisting infections against challenge with RhCMV lacking the US2-11 immunoevasins (42). Therefore, we used a previously described heterologous prime-boost regimen to induce pp65b-specific T cell responses (45, 46). Three animals were vaccinated with DNA encoding pp65b, followed by 2 boosts with pp65b-expressing modified vaccinia Ankara (MVA). For control, we vaccinated 3 animals with antigen-free plasmid and MVA. As shown in Figure 5A (Supplemental Figure 3), all 3 pp65b-vaccinated animals developed a robust CD4+ and CD8+ T cell response to pp65b after this prime-boost vaccination regimen that, in the 6 weeks following the final boost, was similar in magnitude and phenotype to pp65-specific T cell responses that develop in the context of RhCMV infection (Figure 3, iii and iv, and Supplemental Figure 4). As expected, pp65b-specific T cells were not observed in the control MVA-vaccinated group. Six weeks after the final MVA/pp65 versus control MVA boost, all animals were challenged with RhCMV lacking US2-11 and expressing SIVgag (AUS2-11gag). Similar to AIVHCEAUS2-11gag, this virus is unable to overcome preexisting T cell immunity, despite the presence of the RhCMV-specific MHC-I inhibitor VIIHCE (42). All 3 control-vaccinated animals developed the expected T cell response to pp65 as
of Δpp65-infected cells were used as antigen, a very modest antibody response was observed in all animals challenged with ΔUS2-11, and there was no difference in the kinetics or magnitude specificity of this response between pp65-vaccinated and control-vaccinated animals (Figure 5B), and we did not observe a difference in the specific antigens recognized by immunoblot (Figure 5D). Remarkably, when pp65-containing CMV lysate was used as antigen for our ELISA or immunoblot, we observed an extraordinary increase in the titers of pp65-specific antibodies induced by DNA/MVA vaccination. As shown in Figure 5B, pp65-specific antibodies were above background levels upon boosting with MVApp65. Moreover, subsequent challenge with ΔUS2-11 increased these pp65-specific antibodies by several orders of magnitude. pp65 antigen was recognized in sera from pp65-vaccinated animals by immunoblot, and this response was strongly increased upon challenge. In contrast, control-vaccinated animals did not recognize the corresponding 65-kDa band even after ΔUS2-11 challenge (Figure 5D). These data indicate that pp65 vaccination did not affect ΔUS2-11 viral load to a level that would affect the induction of CMV-specific antibodies, although a modest reduction, as reported previously, cannot be ruled out. In addition, these observations suggest that antibody responses to pp65, and potentially to other antigens as well, are substantially boosted upon infection with ΔUS2-11 virus.

**pp65 limits dissemination of RhCMV in vivo.** Taken together, these data suggest that neither pp65 itself nor the T cell response to pp65 have a major impact on the overall course of either primary or secondary RhCMV infection. However, it remained possible that the extent of RhCMV replication is affected by the presence or absence of pp65. If lack of pp65 delays RhCMV replication kinetics in vivo, as it does in vitro, infections with pp65-deleted RhCMV would be expected to proceed more slowly and/or manifest reduced spread or peak viral production relative to WT virus. On the other hand, if pp65-induced immune responses have superior efficacy, infections with pp65-deleted RhCMV would manifest greater viral replication and spread than WT RhCMV. To address these possibilities, we performed experiments in which genetically marked WT and Δpp65ab RhCMV constructs (using SIVgag and SIVretanef as the identifying markers, respectively) were simultaneously, but separately (right arm vs. left arm), inoculated into either RhCMV seropositive (n = 2) or RhCMV seronegative RMs (n = 3). These RMs were sacrificed and taken to necropsy at 14, 21, or 28 days after inoculation; DNA was isolated from the sites of inoculation and distant tissues; and the extent and magnitude of viral spread was determined by an ultrasensitive, nested quantitative PCR analysis using primers that specifically amplify fragments of the SIVgag versus SIVretanef inserts (48). In the setting of superinfection, little tissue-associated viral DNA was detected for either the WT or Δpp65ab constructs, with the former only identified in one of the inoculation sites in the RM analyzed at day 14 and the latter identified at very low level in inoculation sites and scattered distant tissues in both the RMs analyzed at day 14 and day 28 after inoculation (Table 3). Although, in these 2 RMs, the extent of spread by the Δpp65ab RhCMV was greater than that of the coadministered WT RhCMV, the level of tissue-associated virus observed in the Δpp65ab RhCMV superinfection was still within the range of that found in WT RhCMV superinfection in other RMs (Table 4), and thus, deletion of pp65 does not seem to significantly affect viral dissemination during superinfection. In striking contrast, the extent of Δpp65ab RhCMV replication in RhCMV-naive RMs was 3 to 4 logs higher than the simultaneously administered WT RhCMV (Table 5). Indeed, the degree of Δpp65ab RhCMV replication during primary infection was astonishing, reaching almost 10^8 DNA copies in the LN draining the injection site at day 14 after inoculation. Analyses of viral loads in blood indicate that the difference in WT versus Δpp65ab RhCMV replication by can be observed by day 7 after inoculation (Table 5). The levels of Δpp65ab RhCMV in blood and tissue declined dramatically at later time points, indicating that this virus is eventually brought under immune control. Taken together, these data unequivocally indicate that expression of pp65 strongly limits primary viral dissemination over several orders of magnitude and suggest that, while RhCMV-specific T cells can control RhCMV in the absence of pp65, a rapid immune response to pp65 is necessary to limit viral spread during the early days of primary infection.

### Table 4

<table>
<thead>
<tr>
<th>Tissue type</th>
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<th>21 dpi</th>
<th>28 dpi</th>
</tr>
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<tbody>
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<td>47</td>
<td>16,502</td>
<td>376</td>
</tr>
<tr>
<td>Rh25976</td>
<td>541</td>
<td>23,793</td>
<td>4,480,600</td>
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<td>7</td>
<td>355</td>
</tr>
<tr>
<td>Axillary lymph node (mutant)</td>
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<td>7</td>
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<td>3,429</td>
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<td>Axillary lymph node (mutant)</td>
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<td>7</td>
<td>1,600</td>
</tr>
<tr>
<td>Spleen</td>
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<td>1</td>
<td>18</td>
</tr>
<tr>
<td>Liver</td>
<td>6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>1</td>
<td>ND</td>
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<tr>
<td>Brain</td>
<td>3</td>
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</tr>
<tr>
<td>Spinal cord</td>
<td>9</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Genome copy numbers in tissue samples are given per 10^7 cell equivalents, whereas genome copies in urine are shown per ml.
The goal of this study was to assess the role of one of the major structural components of the CMV virion in infection and immunity. Similar to HCMV, a large percentage (approximately 11%) of the protein mass of RhCMV virions consists of the 2 pp65 homologs (38). Nevertheless, in both HCMV and RhCMV, pp65 is not essential for growth in vitro although increased production of defective particles occurs during infection with RhCMVΔpp65ab. This could be reflective of assembly defects due to the lack of pp65ab. For HCMV, it has been reported that pp65 is required for the incorporation of other virion proteins, most notably UL25, UL69, and UL97 (31). However, we did not observe a major skewing in the protein composition of the viral tegument as would have been expected if pp65 selectively controls the incorporation of other viral proteins. Instead, the tegument composition seemed normal but without pp65 present. Conceivably, this could be due to a difference in virion assembly between HCMV and RhCMV. However, the overall virion proteome of RhCMV is highly similar to that of HCMV (38), and UL25, UL69, UL97 are highly conserved in RhCMV. Therefore, it seems more likely that effects of pp65 on incorporation of other tegument proteins are nonselective. The fact that virions are assembled, carrying the same ratios of viral proteins as WT, while lacking pp65, suggests that although pp65 might facilitate virus assembly, once the virus is assembled, the lack of pp65 does not affect the overall viral structure, except for a reduction on overall virion size due to a reduced tegument protein layer.

In addition to viral assembly, pp65 has an immediate function upon release of the tegument into cells during membrane fusion. Similar to other tegument proteins (e.g., pp71 and UL35), pp65 is thought to contribute to setting the stage for optimal viral replication by counteracting intrinsic and innate antiviral host response mechanisms (49). HCMV lacking pp65 showed increased induction of IFN-stimulated genes (ISGs) (19, 20). We reported previously that RhCMV particles inhibited ISG expression (50) and pp65 was a possible candidate for this inhibition. However, Δpp65ab did not induce ISGs, suggesting that inhibitory mechanisms mediated by other RhCMV proteins perform this function.

### Table 5

<table>
<thead>
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<td>560</td>
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<td>Skin injection site (Δpp65ab)</td>
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<td>Axillary lymph node (WT)</td>
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<td>500</td>
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<td>Axillary lymph node (Δpp65ab)</td>
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<td>ND</td>
<td>ND</td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Iliosacral lymph node (Δpp65ab)</td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Inguinal lymph node (WT)</td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Inguinal lymph node (Δpp65ab)</td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Inferior mesenteric lymph nodes</td>
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<td>ND</td>
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<tr>
<td>Superior mesenteric lymph nodes</td>
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<td>Colon</td>
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<td>ND</td>
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<td>67,120</td>
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<td>ND</td>
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</tr>
<tr>
<td>Plasma 28 dpi</td>
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<td>–</td>
<td>ND</td>
</tr>
</tbody>
</table>

Genome copy numbers in tissue samples are given per 10^7 cell equivalents, whereas genome copies in urine and plasma are shown per ml.
research article

... (data not shown). It has also been reported that HCMV pp65 binds to and induces the major immediate early promoter (MIEP) in conjunction with the cellular protein IFI16 (21). We have not investigated in detail the impact of pp65 on IE expression in RhCMV. However, in this study, we did observe a delay in virus production in multistep growth curves (Figure 1C), consistent with this effect. Thus, RhCMV pp65 proteins appear to facilitate optimal expression of viral genes in the early stages of cellular infection but are not required for productive infection.

The role of HCMV pp65 for the establishment and maintenance of infection in vivo is unknown due to the strict species specificity of HCMV. The rationale for studying RhCMV pp65 in the RM model was therefore the close evolutionary relationships of both the host to human and of the virus to HCMV. Given the multiple functions assigned to HCMV pp65, it was completely unexpected that deletion of both homologs in RhCMV did not only not affect the ability of RhCMV to establish and maintain a long-term infection in the rhesus host but, in fact, strongly increased the ability of RhCMV to replicate and disseminate during primary infection. In contrast, lack of the pp65 homologous genes M83/M84 and GP83 in murine CMV (MCMV) and guinea pig CMV, respectively, reduced peak viremia during primary infection (51–53). In contrast to primary infection, differences in the replication and dissemination of App65ab versus WT RhCMV were minimal, if not absent, in the setting of superinfection of CMV' RMs, suggesting that once established, adaptive immune responses to antigens other than pp65 can effectively control the infection. These data suggest that pp65 likely acts as an "immunological brake" during the initial stages of primary infection to limit viral replication and dissemination. We therefore hypothesize that the main function of pp65 is not that of immune evasion, but immune induction, i.e., eliciting a rapid immune response that controls viremia. The most likely candidate mechanism for this effect is the pp65-specific effector T cell response, which, due to the abundance and immunogenicity of pp65 proteins, might appear earlier in primary infection than the response to other CMV proteins. Alternatively or additionally, pp65 might induce innate immune responses that limit viral replication in primary infection. This innate induction function of pp65 is reminiscent of the NK cell–stimulating protein m157 of MCMV, whose deletion or mutation increases viral replication and titers in mice carrying the NK cell receptor Ly49H for which m157 is a ligand (54, 55).

The parental strain used to generate App65ab, RhCMV 68-1, shows reduced secretion from infected animals, most likely due to the lack of genes in the ULb-homology region required for tissue tropism (56). Since RhCMV 68-1 does not generate robust plasma viremia in infected animals, the appearance of RhCMV-App65ab in plasma samples became particularly striking. Thus, it is conceivable that the increased dissemination of App65ab might be less pronounced in viruses carrying an intact ULb region. However, in preliminary observations, we did not observe increased dissemination of RhCMV 68-1,2, a virus that is repaired for tissue tropism (57). Thus, it is likely that pp65 deletion will have a similar effect on a repaired or low-passage viral background, although this still needs to be verified experimentally.

T cells from HCMV-infected individuals recognize a broad spectrum of viral ORFs that are highly variable between individuals (15). Although no ORFs are recognized by all seropositive people, pp65 is one of the most consistently recognized CMV proteins by both CD4+ and CD8+ T cells (15), a level of immunogenicity that has led vaccine developers to include pp65 in HCMV vaccines (11, 58–62). However, relatively little is known about the protective effect of pp65-specific T cells in humans, since vaccine trials generally involve a cocktail of proteins and efficacy cannot be directly correlated to pp65 alone (13). The most direct evidence for a protective effect of pp65-specific T cells comes from adoptive T cell transfer experiments that used pp65-derived peptides to expand HCMV-specific T cells (63–68). In these studies, transfer of pp65-specific T cells accelerated the restoration of anti-viral immunity posttransplantation, without graft versus host side effects associated with nonspecific T cell transfer. Our finding that pp65-specific immunity seems to curtail viral dissemination in the early stages of infection would support the inclusion of pp65 in subunit vaccines, provided it is indeed the pp65-specific T cells that are responsible for this effect.

On the other hand, our data also indicate that pp65-specific T cell responses are not sufficient to recapitulate the level of protective immunity generated by actual viral infection. To examine the protective effect of pp65-specific T cells or T cells specific to other CMV antigens we developed a novel challenge strategy. Our approach relies on our previous observation that viral genes encoding the RhCMV homologs of HCMV immunoevasins US2, 3, 6, and 11 are essential for RhCMV to superinfect RhCMV-positive animals (42, 69). The ability to establish secondary persistent infections is also a common occurrence in HCMV, resulting in frequent coinfection with different strains of HCMV (7). Since depletion of CD8+ T cells restores the ability of US2-11–deleted RhCMV to infect seropositive animals, infection with US2-11 viruses can be used to monitor the quality of a vaccine-induced T cell response. Moreover, the clear protection observed by natural infection allows these studies to take place in a very small group of animals, since the outcome of superinfection is binary. The results shown in Figure 3 are typical: both animals inoculated with App65ab were clearly protected against superinfection with US2-11–deleted RhCMV but not with WT. (In fact, we observed superinfection in more than 200 animals inoculated with recombinant RhCMV.) Evidently, the T cell responses to antigens other than pp65 are protective in this challenge model.

Using the US2-11 challenge approach we were able to examine whether pp65-specific T cells elicited by heterologous prime-boost vaccination were sufficient to recapitulate the protective effect of T cells elicited by preexisting infection. We used a DNA-prime/MVA-boost protocol employed previously to vaccinate animals with a combination of pp65, IE1, and gB (44). In this previous work, it was shown that this vaccination regimen, while unable to protect against superinfection with RhCMV, reduced local and systemic viremia as well as viral shedding. Moreover, reduction in shedding correlated with the magnitude of pp65-specific T cell responses (44). In our hands, the heterologous prime-boost vaccination induced a robust CD4+ and CD8+ T cell response to pp65 that in the blood was similar in magnitude to pp65-specific T cell responses elicited by RhCMV infection. Although prime-boost vaccination would not be expected to maintain the effector-memory–biased T cell responses elicited by RhCMV infection over the long term (41, 48, 69), at the time of challenge (6 weeks after the final MVA boost), the responses generated by the prime-boost vaccine still manifested a predominant effector memory phenotype. Despite this, these vaccine-generated pp65-specific T cell responses were insufficient to protect against infection by US2-11 gag virus, as shown by the induction of Gag-specific T
cell responses and CMV-specific antibody responses. This suggests that T cells induced by pp65 alone do not reproduce the protective effect of T cells induced by ongoing persistent infections. Since the T cell response to pp65 was substantial in all 3 animals, it seems unlikely that a different vaccination strategy would have induced a better protection. Rather, it seems more likely that additional antigens might be required to recapitulate the protective effect of natural infection. Thus, our results caution against the use of pp65 as the only T cell stimulatory subunit in a CMV vaccine.

The ΔUS2-11 challenge used in this study provides an excellent tool to evaluate the T cell component of subunit vaccines. Conceivably, a similar approach could be used in human clinical trials to specifically evaluate the T cell immunity generated by a given vaccine. Recently, challenge with the Towne strain was used to evaluate the efficacy of subunit vaccines by monitoring an anamnestic HCMV-specific immune response (61). Conceivably, a US2-11–deleted Towne strain would not generate an anamnestic response, similar to our observation that IE1-specific responses were not boosted when RhCMVΔUS2-11 failed to superinfect (Figure 3). In this case, a second challenge with WT-Towne could be used to monitor protection and T cell boosting as described previously (61). In contrast, a boosting of the pp65-specific T cell and antibody responses, as observed in pp65-vaccinated animals (Figure 5), would be a clear indication of infection by US2-11–deleted Towne and lack of protection by the T cell component of a given vaccine. Furthermore, our observation that vaccine-induced antibody responses against pp65 were strongly boosted by inoculation with ΔUS2-11 virus suggests that a safe, US2-11–deleted HCMV could be used to boost antibody levels induced by a given vaccine. Since high levels of antibodies are a desired feature of many vaccine regimens, inclusion of CMV as a new tool to enhance antibodies should be considered.

In summary, our work revealed a novel and surprising function of pp65, suggesting that this viral protein acts as an immune inducer that generates an immune response that stringently restricts viral replication during primary infection but that has little impact on long-term maintenance, immunogenicity, or viral shedding. A likely explanation for this finding is that the highly abundant and immunogenic protein pp65 induces a rapid T cell response that limits viral dissemination. Since an intact immune system is required for this “immunological brake” mechanism to function, the lack of pp65-mediated control likely contributes to the high level of dissemination observed in immunocompromised individuals, such as transplant recipients, or in fetuses with immature immune systems. In RMs, RhCMV can cause severe sequelae, including spontaneous abortions, when injected into the developing fetus (70, 71). Since the immune-dominance of pp65 is conserved in HCMV, it is likely that this “antivirulence” function is conserved as well. HCMV and RhCMV thus seem to use the adaptive immune response to limit their dissemination during primary infection. It is not immediately obvious why CMV would choose such a self-imposed restriction. However, since the establishment and maintenance of persistent infection, as well as persistent shedding from the infected host, is not affected by the presence or absence of pp65, it seems that the ultimate “goal” of CMV, to establish a benign infection that easily spreads through the human population, is unaffected by pp65. The pp65-mediated immunological control of CMV dissemination might thus serve to soften the impact of primary infection on the host to ensure a healthy host that is able to maintain and transmit the virus for a long time.

### Methods

**Cells and reagents.** TRFs (72) were maintained in DMEM with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin and were grown at 37°C in humidified air with 5% CO₂.

**Viruses and construction of recombinant mutants.** BAC-derived RhCMV 68-1 (39) was reconstituted by electroporating BAC DNA into TRFs (250 V, 950 μF), and cytopathic effect was observed after 7 to 10 days. Recombinant RhCMV mutants were created by homologous recombination (73, 74) in E. coli strain EL250, which contains heat-inducible λ recombinase (rec) genes and an arabinose-inducible FLP recombinase (75). Bacterial cultures were grown in LB at 30°C until an OD of 0.35 at 600 nm was reached, and the rec genes were induced through heat induction by shaking the culture at 42°C in a water bath for 15 minutes. The bacteria were subsequently chilled on ice for 10 minutes and made electrocompetent by washing them 4 times with cold, deionized water. Electrocopent EL250 were always made and used fresh to increase the recombination efficiency.

To construct the pp65a and pp65b (RhCMV-App65ab) double-deletion virus, recombination primers containing 50 bp homology to regions flanking the pp65 ORFs (forward mutagenesis primer 5′-GAAGATATGTTGCGGTCTCGGGGGATTGGGGTTTTTATATAGGTATGGGT-3′ and reverse mutagenesis primer 5′-ATGAGCCAAGTTGCGCACGCTCAGTGGGCGGTGTCGCAAAGTCAAGACAC-3′) were used to amplify a kanamycin (Kan) resistance cassette from plasmid pCP015 (76). The pCP015 forward primer binding site (5′gtaaaacgacggccagt) and reverse primer binding site (5′gaacagaccgaggcag) were added to the 3′ end of the mutagenesis primers.

Competent EL250 bacteria containing WT RhCMV BAC were then electroporated with the PCR product for recombination using a MicroPulser (Bio-Rad) and selected for Kan and chloramphenicol (Cm) resistance at 30°C on LB agar. To induce the FLP recombinase excising the Kan cassette, clones were grown in LB with Cm until they reached an OD of 0.5 at 600 nm and incubated with 1 mg/ml arabinose for 1 hour. The bacteria were streaked out on an LB plate with Cm selection using an inoculation loop and incubated overnight at 30°C. After colonies were visible, clones were replica plated on LB agar with Kan and Cm and LB agar with Cm only, and colonies were selected that had lost Kan and characterized by restriction digest, Southern blot, and partial sequencing. Virus was reconstituted by electroporation of TRFs with 5 to 10 μg of BAC DNA.

To construct the second pp65ab double-deletion mutant containing the SIV ref/tat/nef (retanef) fusion protein, which was driven by the EF1α promoter and inserted in place of the pp65ab genes and an arabinose-inducible FLP recombinase (75). Bacterial cultures were grown at 37°C in humidified air with 5% CO₂.

**RhCMV particle purification procedures.** RhCMVApp65ab virions were purified over a discontinuous Nycodenz gradient, as described before for HCMV AD169 (18) and RhCMV 68-1 BAC-derived WT (38). The virus was isolated from the culture medium of infected TRFs when the cells displayed maximal cytopathic effect. The cellular supernatants were first clarified by centrifugation at 7,500 g for 15 minutes. The clarified medium was layered over a sorbitol cushion (20% D-sorbitol, 50 mM Tris [pH 7.4], 1 mM
MgCl	extsubscript{2}), and virus was pelleted by centrifugation at 64,000 g for 1 hour at 4°C in a Beckman SW28 rotor. The virus pellet was resuspended in TNE buffer (50 mM Tris [pH 7.4], 100 mM NaCl, and 10 mM EDTA). The virus particles were further purified by layering them over a discontinuous 5% to 50% Nycodenz gradient (Sigma-Aldrich) in TNE buffer and centrifuging at 111,000 g for 2 hours at 4°C in a Beckman SW41 Ti rotor. The virion bands in the gradient were isolated with a syringe through the side of the centrifuge tube, and the particles were pelleted in a Beckman TLA-45 rotor in a Beckman Optima TL 100 Ultracentrifuge at 40,000 g for 1 hour and washed twice with TNE buffer. The pellet was resuspended in TNE buffer, and electron microscopy was performed to confirm the purity of the sample. In order to assess the protein content of the purified RhCMVApp65ab virions, especially in comparison to a parental RCMV WT sample, a denatured protein preparation was separated on a NuPAGE morpholine propanesulfonic acid (MOPS) gradient gel (Inn Vivogen) and visualized by Coomassie brilliant blue staining (Figure 2C).

Quantitative proteomic analysis. The quantitative proteomic analysis was performed as previously described in detail (38). Briefly, RhCMV-derived particles were denatured in 8 M urea, 100 mM NH	extsubscript{4}HCO	extsubscript{3}, and 5 mM DTT, and the cysteine residues were alkylated with 10 mM iodoacetamide. Then, the samples were 4-fold diluted with 25 mM NH	extsubscript{4}HCO	extsubscript{3} and 1 mM CaCl	extsubscript{2} and digested overnight with a 1:20 (mass/mass) trypsin-to-protein ratio. The digested peptides were desalted with C18 cartridges and dried in a vacuum centrifuge before being separated in capillary columns (75 μm × 65 cm capillary [Polymicro] packed with 3-μm C18 particles [Phenomenex]) connected to a custom-made 4-column liquid chromatography LC system (77) or a longer capillary column (75 μm × 100 cm) connected to a nanoAcquity system (Waters). Eluting peptides were analyzed directly in a linear ion-trap orbitrap mass spectrometer (LTQ Orbitrap XL, Thermo Scientific).

Collected MS/MS spectra were searched against forward and reverse sequences of the RhCMV ORFs (275 sequences), Macaca mulatta Ensembl database (21,905 sequences, downloaded from http://www.ensembl.org on November 15, 2010), and 186 common contaminants (downloaded from http://www.peptideatlas.org; dataset identifier: PASS00367).

**RMs.** A total of 9 male and 4 female purpose-bred juvenile RMs (M. mulatta) of Indian genetic background were used in this study. All RMs were specific-pathogen free (SPF), as defined by being free of cercopithecine herpesvirus 1, D-type simian retrovirus, simian T lymphotrophic virus type 1, SIV, rhesus rhadinovirus, Mycobacterium tuberculosis, and RhCMV infection at the start of the study. The Δpp65ab-deleted RhCMV vector was tested in vitro by administering the deltavirus s.c. at a dose of 1 x 10^7 PFUs to 2 SPF (defined above) RMs. To test whether the immune responses generated by the primary infection with Δpp65ab were sufficient to prevent superinfection, the same 2 RMs were inoculated with the same vaccine strategy using pND (empty) and MVA (empty), respectively (45, 46). The plasmids pND and pND/pp65ab were provided by Peter A. Barry, UCID, Davis, California, USA (45); the empty MVA as well as the pp65ab-expressing recombinant MVA were provided by Don J. Diamond, City of Hope, Duarte, California, USA. As a first step, the gene encoding RhCMV pp65 was amplified from previously described plasmid expression vectors and engineered into the pZWIIA MVA transfer vector using established protocols (46). rMVA expressing RhCMV pp65 was generated on BHK-21 cells via homologous recombination. The protein expression levels for RhCMV pp65 in infected BHK-21 cells were confirmed by Western blot using polyclonal antibodies to RhCMV pp65-2 by chemiluminescence detection (ECL, Amersham Pharmacia Biotech). The plasmid DNA of pND and pND/pp65a was isolated using the EndoFree Plasmid Mega Kit (Qiagen) to avoid any endotoxin contamination of the DNA. Vaccinated animals were challenged s.c. 18 weeks after the initial DNA vaccination with 10^7 PFUs RhCMV ΔUS2-11gag.

BAL fluid, peripheral blood, and urine samples were collected at specified time points (see Figures 3–5) throughout the entire experiment. Isolated CD4^+ and CD8^+ T cells from BAL fluid and peripheral blood were stimulated with antigen-specific peptides to examine the immune response induced by the initial vaccination and the subsequent viral challenge.

**Nested real-time PCR.** To determine the viral copy numbers of RhCMV 68-1 GAG and RhCMVApp65ab retaief, 3 naive and 2 CMV^+ RM s were infected s.c. with 10^7 PFUs of each virus in the opposite arm on the same day. Blood samples were taken once a week to monitor CD4^+ and CD8^+ T cell responses and to determine the presence of cell-free virus in PBMCs. At the indicated time points after infection, the macaques were necropsied and tissues were harvested. DNA was isolated from the samples by ONPRC’s Molecular Virology Support Core (MVSC). Tissue samples (~100 mg) were prepared using Fastprep (MP Biomedicals) in 1 ml TrizReagent (Molecular Research Center Inc.). 100 μl bromochlorophenol (MRC Inc.) was added to each homogenized tissue sample to enhance phase separation. 0.5 ml DNA back-extraction buffer (4 M guanidine thiocyanate, 50 mM sodium citrate, and 1 M Tris) was added to the organic phase and interphase materials, which was then mixed by vortexing. The samples were centrifuged at 14,000 g for 15 minutes, and the aqueous phase was transferred to a clean microfuge tube containing 240 μg glyoxigen and 0.4 ml isopropanol and centrifuged for 15 minutes at 14,000 g. The DNA precipitate was washed twice with 70% ethanol and resuspended in 100 to 500 μl ddH2O. Nested real-time PCR was performed with primer and probe sets for the inserted SIV proteins GAG (first round: for-GAAAACATGCGAAGACCTCCTC and rev-CTCGTTGTAGTAGTGACGGGATG; second round: for-CAACCTACGTCACGTCAGCCTGCTC, rev-TCAACAGCGATTCGACAGCTCGG, and probe-CGGAGAAAGCTTGGGTCAAGCFFAM) and SIVretanef (first round: for-CAACGAGGAGGACGGAGACGGGACAGG; second round: for-CAACGAGGAGGACGGAGACGGGACAGG, rev-CAACGAGGAGGACGGAGACGGGACAGG, and probe-CTCCTCTCTCTCTGTGGATGAGCTCAGCATCGCAGTFAM). For each DNA sample, 10 individual replicates (5 μg each) were amplified by first-round PCR synthesis (12 cycles of 95°C for 30 seconds and 60°C for 1 minute) using Platinum Taq in 50 μl reactions. Then, 5 μl of each replicate was analyzed by nested quantitative PCR (45 cycles of 95°C for 15 seconds and 60°C for 1 minute) using FastAdvanced Master Mix (ABI Life Technologies) in an ABI StepOnePlus Real-Time PCR machine. The results for all 10 replicates were analyzed by Poisson distribution and expressed as copies per cell equivalents (80).

**Viral detection in urine by culture.** We centrifuged filter-sterilized (0.4 mm) urine at 16,000 g for 1 hour at 4°C to concentrate virus for coculture on rhesus fibroblasts. Cell lysates were prepared after we observed extensive cytopathic effects or after 42 days of coculture if cytopathic effects were minimal or not observed. The prepared cell lysates were assessed for the presence of RhCMV on the basis of expression of RhCMV^+ or SIV-specific antigens by Western immunoblotting.
**Immunologic assays.** RhCMV- and SIV-specific CD4+ and CD8+ T cell responses were measured in mononuclear cell preparations from blood and BAL fluid by flow cytometric intracellular cytokine analysis, as previously described (41). Briefly, sequential 15-mer peptides (overlapping by 11 amino acids) comprising the SIVmac239Gag, or Rev/Nef/Tat and RhCMV 68.1p66sab, or EI1/2 were used in the presence of costimulatory CD28 and CD49d monoclonal antibodies (BD Biosciences). Cells were incubated with antigen and costimulatory molecules alone for 1 hour, followed by addition of Brefeldin A (Sigma-Aldrich) for an additional 8 hours. Costimulation without antigen served as a background control. Cells were then stained with fluorochrome-conjugated monoclonal antibodies, flow cytometric data were collected on a LSR II (BD Biosciences), and data were analyzed using the FlowJo software program (version 8.8.7; Tree Star). Responses frequencies (CD69+TNFα and/or CD69+IFNγ) were first determined in the overall CD4+ and CD8+ population and then memory corrected (with memory T cell subset populations delineated on the basis of CD28 and CD95 expression).

**Measuring RhCMV-specific antibody responses by ELISA.** Antibody levels to RhCMV were measured in circulating plasma of RMs by standard ELISA using plates coated with lysates of fibroblasts infected with either WT-RhCMV or RhCMV-App65 at 10 ng total protein per well. Nonspecific binding sites were blocked with 2% milk powder resuspended in PBS. Serial 2-fold dilutions of plasma were incubated for 1.5 hours prior to washing 3 times with ELISA wash buffer (PBS with 0.1% Tween-20; 200 µl per well). Primary antibody binding was detected and quantified with HRP-conjugated anti-rhesus IgG/IgA/IgM secondary antibody and addition of o-phenylenediamine chromogen substrate. A log-log transformation was performed on the linear portion of the curve and end point titers were calculated using 0.1 OD units as the cutoff point. Each plate contained a positive control sample to normalize ELISA titers between assays and a negative control sample to ensure assay specificity and to subtract background. Graphical data was generated using Prism GraphPad software.

**Measuring RhCMV-specific antibody responses by SDS-PAGE and Western blotting.** Rhesus fibroblasts infected with WT-RhCMV or RhCMV-App65 were solubilized in 2x Laemmli’s sample buffer, and 200 µg total protein per lane were loaded onto NuPAGE 4%–12% Bis-Tris gradient gels (Invitrogen, Grand Island, NY). Samples were run in MOPS buffer. Proteins were transferred to Immobilon-P blotting membrane, and nonspecific binding sites were blocked in 2% milk powder in Tris-buffered saline with 0.02% Tween-20. Membranes were incubated with antigen and costimulatory molecules alone for 1 hour, followed by addition of Brefeldin A (Sigma-Aldrich) for an additional 8 hours. Costimulation without antigen served as a background control. Cells were then stained with fluorochrome-conjugated monoclonal antibodies, flow cytometric data were collected on a LSR II (BD Biosciences), and data were analyzed using the FlowJo software program (version 8.8.7; Tree Star). Responses frequencies (CD69+TNFα and/or CD69+IFNγ) were first determined in the overall CD4+ and CD8+ population and then memory corrected (with memory T cell subset populations delineated on the basis of CD28 and CD95 expression).

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Address correspondence to: Louis J. Picker or Klaus Früh, Vaccine and Gene Therapy Institute, Oregon Health and Science University, 505 NW 185th Ave., Beaverton, Oregon 97006, USA. Phone: 503.418.2735; Fax: 503.418.2701; E-mail: pickerl@ohsu.edu (L.J. Picker), fruehk@ohsu.edu (K. Früh). Ernesto S. Nakayasu’s present address is: Bindley Bioscience Center, Purdue University, West Lafayette, Indiana, USA.

Kasinath Viswanathan’s present address is: Advinus Therapeutics Ltd., Hinjewadi, Pune, India.

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