The gammaherpesvirus 68 viral cyclin facilitates expression of LANA

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Abstract

Gammaherpesviruses establish life-long infections within their host and have been shown to be the causative agents of devastating malignancies. Chronic infection within the host is mediated through cycles of transcriptionally quiescent stages of latency with periods of reactivation into detectable lytic and productive infection. The mechanisms that regulate reactivation from latency remain poorly understood. Previously, we defined a critical role for the viral cyclin in promoting reactivation from latency. Disruption of the viral cyclin had no impact on the frequency of cells containing viral genome during latency, yet it remains unclear whether the viral cyclin influences latently infected cells in a qualitative manner. To define the impact of the viral cyclin on properties of latent infection, we utilized a viral cyclin deficient variant expressing a LANA-beta-lactamase fusion protein (LANA::βla), to enumerate both the cellular distribution and frequency of LANA gene expression. Disruption of the viral cyclin did not affect the cellular distribution of latently infected cells, but did result in a significant decrease in the frequency of cells that expressed LANA::βla across multiple tissues and in both immunocompetent and immunodeficient hosts. Strikingly, whereas the cyclin-deficient virus had a reactivation defect in bulk culture, sort purified cyclin-deficient LANA::βla expressing cells were fully capable of reactivation. These data emphasize that the γHV68 latent reservoir is comprised of at least two distinct stages of infection characterized by differential LANA expression, and that a primary function of the viral cyclin is to promote LANA expression during latency, a state associated with ex vivo reactivation competence.

Author summary

Gammaherpesviruses are ubiquitous viruses with oncogenic potential that establish latency for the life of the host. These viruses can emerge from latency through reactivation, a process that is controlled by the immune system. Control of viral latency and reactivation is thought to be critical to prevent γHV-associated disease. This study focuses on a virally-encoded cyclin that is required for reactivation from latency. By characterizing how the viral cyclin influences latent infection in pure cell populations, we find that the
viral cyclin has a vital role in promoting viral gene expression during latency. This work provides new insight into the function of a virally encoded cyclin in promoting reactivation from latency.

Introduction

Gammaherpesviruses (γHV) are a group of lymphotropic viruses within the Herpesviridae family, including the human pathogens Epstein-Barr virus (EBV) and Kaposi’s sarcoma-associated herpesvirus (KSHV, HHV-8) and the mouse pathogen Murine gammaherpesvirus 68 (γHV68, MHV68, MuHV4). Infection with these viruses can result in development of a wide range of malignancies including Burkitt’s lymphoma, Kaposi’s sarcoma, nasopharyngeal carcinoma, post-transplant lymphoproliferative disorders, and primary effusion lymphoma [1,2]. The mouse gammaherpesvirus, γHV68, is closely related to both EBV and KSHV, readily infects laboratory strains of mice, and provides insights into the complex processes of γHV pathogenesis [3,4].

γHV infection can be characterized by two distinct phases, lytic and latent infection. Lytic infection is a productive form of infection in which there is widespread transcription and translation of viral genes and the virus actively replicates its genome [5–7]. In this process, new virus is produced and the lytically infected cell dies. Alternatively, the virus may enter a latent state of infection, in which viral gene expression is mostly suppressed and the viral genome is maintained as an episome in the host nucleus [8]. γHV are able to switch from latent to lytic infection through a process known as reactivation [9, 10]. These viruses are able to establish latent infection in many different cell types, and in γHV68, including dendritic cells, macrophages, and multiple B cell subsets (including memory B cells, plasma cells, B1-a cells, and B1-b B cells), with germinal center and memory B cells representing the major latently infected cell populations [11–17]. Although several cell types support latent infection, the relative efficiency of these cell types to support reactivation remains unknown. Numerous studies suggest that, in lymphoid tissues, a primary source of reactivating virus is plasma cells [14,18–20]. Notably, however, studies indicate that in the peritoneal compartment, infected macrophages and/or B1 B cells are major cell types capable of reactivation [12,13].

Many viral and host factors contribute to the control of latent infection and reactivation. KSHV and γHV68 both encode a conserved viral cyclin (v-cyclin), which is homologous to host D-type cyclins [3,21,22]. Although EBV does not encode its own cyclin, it expresses viral genes that upregulate host cyclin D2, fulfilling a similar function to the KSHV and γHV68 v-cyclin [23]. Like the host cyclins, the v-cyclin has the ability to interact with host cyclin-dependent kinases (CDKs) and promote cell cycle progression [24,25]. Unlike conventional host cyclins, the v-cyclin is resistant to inhibition by CDK inhibitors (CKI) [26]. Recent work by our group showed that one mechanism by which the v-cyclin promotes reactivation is by antagonizing the host CKI p18Ink4c, in a cell intrinsic manner [27,28].

Although the v-cyclin is required for reactivation from latency, the specific qualities conferred by the v-cyclin to promote reactivation have yet to be elucidated. Here, we studied how the v-cyclin may influence latent gene expression in vivo, through the use of recombinant γHV68 viruses that encode a fusion of the ORF73/LANA latency-associated gene with β-lactamase, a robust enzymatic reporter gene that can be used to identify individual virally-infected cells [28,29], referred to as LANA::βla. By comparing wild-type and cyclin-deficient viruses, we were able to quantify the frequency and cellular distribution of LANA::βla gene expression during latency. These studies demonstrate that the v-cyclin has a critical role in promoting
expression of LANA:βla at the single-cell level, with no discernable impact on the cellular distribution of infection. Further, we find that the v-cyclin is completely dispensable for reactivation, when reactivation is assessed in LANA:βla expressing cells. The work detailed here serves to further our understanding of how the virus regulates reactivation. We also highlight an emerging trend in the field of virology where latency may not be uniformly defined or refer to a homogeneous state of infection. Rather, some latently infected cells are poised for reactivation, while other infected cells appear to be relatively inefficient or refractory to reactivation.

Results

A cycKO virus expressing a fusion between LANA and β-lactamase is equivalent to wild-type virus in LANA:βla expression during lytic infection, but deficient in reactivation

The v-cyclin is required for γHV68 reactivation. Virus lacking v-cyclin, cycKO, is equivalent to wild-type virus in replication and establishment of latency, but is selectively defective in reactivation from latency [30,31]. Given that some cell types may be more permissive to reactivation from latency than others, we proposed that the cycKO virus may be enriched in, or limited to, a “less permissive” cell type. To address this, we made use of two previously described enzymatically marked viruses, WT.βla and cycKO.βla [28,29]. These viruses both contain a fusion protein where β-lactamase is fused to the viral LANA (Fig 1A). This can be used to

![Image](https://doi.org/10.1371/journal.ppat.1010019.g001)
efficiently identify infected cells by flow cytometry using LANA::βla expression as a surrogate indicator of virus infection. Fusion of β-lactamase to LANA does not appear to alter viral replication, establishment of latency, or reactivation from latency [28,29,32]. To confirm this reporter system works equivalently for the WT,βla and cycKO,βla viruses, we measured the frequency and expression of LANA::βla after lytic infection of mouse 3T12 fibroblasts. 3T12 cells were infected at an MOI of 10 with WT (unmarked), WT,βla, or cycKO,βla virus. At 12 hours post infection (hpi), cells were collected, and stained for β-lactamase activity using CCF2-AM, a cell-permeable β-lactamase substrate [28,29,32]. CCF2-AM is readily taken up by living cells, causing them to fluoresce at 520nM. If β-lactamase is present, indicating viral LANA expression, it then cleaves the substrate causing the cells to gain fluorescence emission at 448 nM. As expected, WT,βla and cycKO,βla viruses resulted in comparable frequency and expression of LANA::βla (βla+) following in vitro infection (Fig 1B). We next confirmed that, as reported, the β-lactamase marker did not alter reactivation phenotypes of either WT or cycKO viruses. C57BL/6 (B6) mice were infected with 1x10⁶ PFU of either WT,βla or cycKO,βla virus via intraperitoneal injection (IP). At 42 days post infection (dpi), splenocytes and peritoneal cells were collected and subjected to limiting-dilution reactivation analysis on permissive mouse embryonic fibroblasts (MEFs) as previously described [12,33]. Briefly, bulk, latently infected splenocytes and peritoneal cells were plated on MEFs. If latent virus reacts, the resulting virions infect and lyse the MEF monolayer. The frequency of latently infected cells can then be determined through nonlinear regression analysis. As previously established in comparison of WT and cycKO viruses in absence of the β-lactamase fusion, the cycKO,βla virus was severely defective in reactivation from both splenocytes and peritoneal cells (Fig 1C). Taken together, these data support the previous reports that fusion of β-lactamase to LANA does not alter the biology of these viruses [28,29,32].

The cell composition of cycKO,βla infected mice is not altered compared to WT,βla infection

To determine if the cycKO virus altered cellular distribution in a particular subset(s) of cells, we infected (B6) mice with 1x10⁶ PFU of either WT,βla or cycKO,βla via IP injection. Splenocytes were harvested at 8 dpi and 16 dpi. Eight dpi is a time point within the acute phase of infection, while 16 dpi corresponds to the establishment of latency after acute infection has been resolved [34,35]. After collection, splenocytes were stained for LANA::βla, CD19, IgD, CD38, and CD44. These markers were used to identify B cells (CD19+), including germinatal center B cells (CD19+, IgD+, CD38-) or activated B cells (CD19+, IgD-, CD44+), as described in Nealy et al [29]. We chose to measure these populations because germinatal center B cells represent an important population for γHV68 to infect and seed memory B cells [36], the primary cell type harboring long-term latent virus, and activating B cells has been show to stimulate reactivation [37]. This staining panel represents the majority of infected cells, and while there are likely other cell types infected, the broad emission of the bla substrate limits the number of fluorophores that can be used. We determined the composition of infected cells by identifying cells expressing the viral LANA::βla fusion protein (Fig 2A). We quantified germinatal center B cells and activated B cells by sequentially gating on CD19+, IgD+, and CD38- or CD44+ respectively (Fig 2B). We saw no significant differences in the expression of these markers on total or infected (βla+) splenocytes at 8 dpi during acute infection (Fig 2C) or at 16 dpi during latency (Fig 2D). In agreement with this, there were no differences between WT,βla or cycKO,βla virus in the frequency of βla+ cells that were total B cells, germinatal center B cells, or activated B cells (Fig 2E). Contrary to our initial prediction, these data suggest that although the cycKO,βla virus is defective in reactivation there are no appreciable differences in the composition of
Fig 2. Cyclin deficient virus has a similar cellular distribution to wild-type γHV68 during primary infection of C57BL/6J mice. Analysis of the cellular distribution of WT and cycKO γHV68 infection in splenocytes from C57BL/6J mice at either 8 dpi (A-C, E) or 16 dpi (D, E), defined based on the distribution of LANA::βla+ cells quantified by flow cytometric analysis. Representative gating of (A) LANA::βla+ splenocytes and (B) B cell subsets, including identification of total B cells (CD19+), germinal center B cells (CD19+, IgD-, CD38-), and activated B cells (CD19+, IgD-, CD44+) at d8 post-infection. (C) and (D) representative histograms of cell surface marker expression after infection with WT.βla or cycKO.βla virus on total cells (dark grey WT.βla and light grey cycKO.βla), WT.βla infected LANA::βla+ cells (black), or cycKO.βla infected LANA::βla+ cells (red), at either 8 dpi (C) or 16 dpi (D). (E) Quantification of the frequency of cells expressing cell surface markers using the gating strategy outlined in (B) with SEM shown, at either 8 dpi (left) or 16 dpi (right). All populations identified were CD19+ B cell subsets; IgD- refers to the frequency of CD19+ B cells that were IgD-; CD38- refers to the frequency of CD19+ IgD- B cells that were CD38 negative; CD44+ refers to the frequency of CD44+. 
CD19+ IgD- B cells that were CD44 positive. 8 dpi CD19, IgD and CD38 n = 13.8 DPI CD44 n = 7. 16 DPI n = 11. Two-tailed student t tests were performed to measure statistical significance. Additional results comparing the frequency of LANA::βla+ cells between WT and cycKO are presented in Fig 3.

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the infected cells compared to WT.βla virus in the cell types analyzed. Thus, there must be another explanation for the reactivation defect observed in v-cyclin deficient viruses.

CycKO.βla virus infection results in deficient expression of viral LANA compared to WT.βla

Splenocytes, from mice infected as above, were collected at 8 and 16 dpi and analyzed by limiting-dilution nested PCR to measure the frequency of splenocytes harboring viral DNA [12,33]. We found that there was a minor decrease in the number of cells harboring cycKO.βla virus at 8 dpi but no significant difference in the number of cells containing γHV68 DNA at 16 dpi (Fig 3A). These data indicate that the reactivation defect in cycKO virus is not due to fewer cells becoming infected, consistent with previously published reports [28,30]. However, when splenocytes were analyzed for the frequency of LANA::βla expressing cells, we found a significantly lower (3.5-fold) frequency of LANA::βla+ cells in mice infected with cycKO.βla (0.06%) compared to wild-type virus infected samples (0.21%) at 8 dpi. Further, this trend continued with 0.03% of splenocytes at 16 dpi that were LANA::βla+ after WT.βla infection compared to 0.008% (3.8-fold decrease) of splenocytes after cycKO.βla infection (Fig 3B). This difference in frequency translated to a decrease in the total number of LANA::βla+ splenocytes per mouse after infection with the cycKO.βla virus (Fig 3C). Considering an equivalent number of cells are viral DNA positive (Fig 3A), this indicates that there is a decrease in the proportion of infected cells that expressed LANA in the absence of v-cyclin. This decreased frequency of LANA::βla+ cells that are B cells, germinal center B cells, or memory B cells translated into a sharp decline in the number of LANA::βla+ cells in cycKO.βla infected mice compared to WT.βla infected mice across multiple subsets (Fig 3D). Consistent with previous analyses, more than 80% of infected cells bore markers consistent with germinal center cells [29]. As WT.βla and cycKO.βla viruses had comparable β-lactamase expression during lytic infection of 3T12 cells (Fig 1B), these data indicate that the v-cyclin promotes the frequency of LANA expressing cells during latent infection in vivo.

The defect in LANA expression with cycKO.βla infection is observed regardless of the tissue type

While we consistently observed a decrease in the frequency of cells expressing LANA::βla after cycKO.βla infection, it remained possible that this was a tissue-specific phenotype. To address this possibility, mice were infected intraperitoneally (IP) as described above and peritoneal cells were collected at 8 and 16 dpi, stained for β-lactamase, CD19, and CD5. CD19 was used to distinguish between non-B cells and B cells (CD19+) and CD5 expression on CD19+ cells was used to identify B1-a cells, which are known to harbor latent virus in the peritoneum (Fig 4A) [13,28]. We saw no significant difference in the cellular distribution of infection between WT and cycKO viruses (Fig 4B), but a pronounced decrease in the frequency of LANA::βla+ cells in peritoneal cells harvested from cycKO.βla infected mice (Fig 4C). There was a significantly lower frequency of LANA::βla+ peritoneal cells after cycKO.βla infection at both 8 (1.7-fold) and 16 dpi (3.3-fold) (Fig 4D). This indicates that the v-cyclin is required for optimal LANA expression in the peritoneum and the spleen, two dominant sites for latency. Finally, to determine whether this effect was dependent on route of infection, we measured the frequency...
Fig 3. Disruption of the viral cyclin has no effect on the frequency of viral genome positive cells yet results in a reduced frequency of LANA::βla+ cells. Mice were infected via IP injection with WT::βla or cycKO::βla viruses and splenocytes were harvested at 8 or 16 dpi. (A) Limiting-dilution nested PCR of viral gene ORF 50 from WT::βla or cycKO::βla infected splenocytes. n = 4 (8 dpi) or n = 3 (16 dpi) with 3–5 mice pooled per group with SEM shown. Comparisons between the LogEC(63.2) found statistical difference between the of WT::βla and cycKO::βla at 8 dpi only (p = 0.009). (B) Representative pseudocolor plots identifying LANA::βla+ splenocytes, indicated in the upper right polygon. Average frequencies of LANA::βla+ cells +/- SEM is indicated below the gate. 8 dpi is shown on the left, while 16 dpi is shown on the right, with WT::βla infected mice on top and cycKO::βla infected mice on bottom. (C) Percent of LANA::βla+ cells (top) and total number of LANA::βla+ splenocytes (bottom) from each individual mouse plotted with SEM shown after infection with WT::βla (black) or cycKO::βla (red) virus. Cells were stained and gated as in Figure 2.8 dpi: LANA::βla+, CD19, IgD, and CD38 n = 13 and CD44 n = 7.16 dpi n = 11. Two-tailed student t tests were performed to measure statistical significance in C and D.

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of LANA::βla+ cells in the lungs at 8 days post-intranasal infection (S1 Fig). Here, mice infected with cycKO.βla virus had a slightly reduced frequency and number of LANA::βla+ compared to WT.βla infected mice. These data demonstrate that the v-cyclin is required for optimal LANA expression, regardless of tissue or route of infection.

The v-cyclin promotes the frequency of LANA expressing cells in immunodeficient, CD8-deficient mice

The v-cyclin is required for optimal reactivation across both immunocompetent and immunodeficient genetic backgrounds [30,33,38]. CD8-deficient (CD8-/-) mice, which lack CD8 T cells, have a significant increase in the number of latently infected cells relative to B6 controls [39]. Despite the overall increase in the number of latently infected cells, the cycKO.βla virus is still defective in reactivation in these mice [33]. We therefore tested whether the v-cyclin was required to promote LANA expression in CD8-/- mice.

CD8-/- mice were infected via IP inoculation with either WT.βla or cycKO.βla virus. Splenocytes were harvested at 16 dpi and stained for β-lactamase activity, CD19 expression, and IgD and CD38 expression on CD19+ cells. We found that, as with B6 mice, there were no differences in cellular distribution of LANA::βla+ between WT and cycKO viruses (Fig 5A and 5C).
Importantly, the defect in LANA::βla expression in cycKO infected splenocytes is still maintained, with a 5.6-fold decrease in the frequency of splenocytes that are βla+ after cycKO.βla infection (0.0034%) compared to WT.βla infection (0.019%) (Fig 5B). We also analyzed peritoneal cell infection at 16 dpi. Peritoneal cells from mice infected as above were collected and stained for β-lactamase activity, CD19, B220, and CD5. The cycKO.βla defect was also present in the peritoneal compartment, with only 0.054% of peritoneal cells LANA::βla+ in cycKO infected samples compared to 0.496% LANA::βla+ cells after WT.βla infection (9.2-fold decrease) (Fig 6A and 6B). We detected a modest shift in the peritoneal composition of LANA::βla+ cycKO.βla infected cells compared to WT infection: 25% of cycKO.βla infected LANA::βla+ cells were CD19+ compared to 12% of WT.βla infected LANA::βla+ cells (Fig 6C). This difference mirrors a change in the total frequency of CD19+ cells in the peritoneum after cycKO.βla infection (Fig 6C). When analyzing the composition of infected B cells by B220 and CD5 expression, the LANA::βla+ cells were found in B1-a, B1-b, and B2 cells, with a higher prevalence in B1 populations. Of WT.βla infected LANA::βla+ cells: 4% were B2 cells, 5% were B1-a cells, and 8% were B1-b cells. Of the cycKO.βla infected LANA::βla+ cells: 10% were B2 cells, 8% were B1-a cells, and 10% were B1-b cells (Fig 6C). Notably, we have previously identified a similar trend in p18Ink4c deficient mice, a mouse strain in which there is an overall increase in reactivation [28], similar to the CD8−/− mice.

Fig 5. Disruption of the viral cyclin results in a reduced frequency of LANA::βla expressing cells in the spleen of CD8−/− mice. At 16 dpi, splenocytes were collected and stained for β-lactamase activity and cell surface markers CD19, CD38, and IgD. CD38 and IgD samples have been previously gated on CD19+ cells. (A) Representative pseudocolor plots are shown identifying LANA::βla+ cells (top row). LANA::βla+ cells are found within the upper right polygon with the average percent of cells expressing LANA::βla +/- SEM indicated below the gate. Expression of CD19, IgD, and CD38 on total cells after WT.βla virus or cycKO.βla virus infection is shown in the indicated columns. Expression of CD19, IgD, and CD38 on LANA::βla+ cells is shown in the indicated columns. The average percent of cells that fall within each gate is indicated +/- SEM. (B) Graphical representation of the percent of cells that are LANA::βla+ after WT.βla (black) or cycKO.βla (red) infection with SEM. (C) Graphed are the average percent of cells that are CD19+ and the percent of CD19+ cells that are IgD+/CD38+, IgD+/CD38+, IgD-/CD38+, and IgD-/CD38- after WT.βla (black) or cycKO.βla (red) infection. Total cells are graphed on the top while LANA::βla+ gated cells are shown on the bottom, both with SEM plotted. Two experiments were performed with 3–4 WT.βla and cycKO.βla infected mice per experiment. WT.βla: n = 7; cycKO.βla: n = 6.

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To determine LANA::βla gene expression independent of enzymatic activity, we isolated WT or cycKO infected peritoneal cells from CD8⁻/⁻ mice and measured both β-lactamase and LANA RNA by qRT-PCR (Fig 6D). Similar to analysis by enzymatic activity, these data demonstrate a difference in latent gene expression at the RNA level between WT and cycKO infected cells at 16 dpi. These data indicate that the v-cyclin is required for optimal LANA gene expression during latency in multiple tissues and in both immunocompetent and immunodeficient hosts.

**LANA expressing cells purified from WT or v-cyclin deficient infection defined by single-cell RNA sequencing**

Our data to this point identify a key role for the viral cyclin in promoting the frequency of latently infected cells expressing LANA. We next sought to interrogate whether there were additional defects present in v-cyclin deficient LANA+ cells using single-cell RNA sequencing. To do this, we FACS purified LANA+ peritoneal cells from CD8⁻/⁻ mice at 16 days post-infection from WT or cycKO-infected mice and subjected cells to 10x Genomics-based 3’-based single cell sequencing. Following quality control and filtering analysis, WT and cycKO LANA+ cells had a comparable number of genes (features) and unique molecular identifiers (UMIs, counts), with the vast majority of cells characterized by low mitochondrial RNA-derived reads and infrequent γHV68-derived reads (Fig 7A–7D). Seurat-based clustering and dimensionality reduction by UMAP further demonstrated that WT and cycKO LANA+ cells had a high degree of overlap (Fig 7E), with cells broadly stratified into a Csf1r expressing (Fig 7F) and a Cd19 expressing (Fig 7G) cells, consistent with a predominant myeloid population and a minor B cell population (Fig 7H). Clustering analysis identified 6 clusters across these two samples (Fig 7I), dominated by myeloid clusters (clusters 1–4, in blue, Fig 7I) with a lower frequency B cell cluster (cluster 5, in orange, Fig 7I). Overall, the cluster frequency between WT and cycKO LANA+ cells was relatively comparable, consistent with our earlier observations that the v-cyclin does not appreciably affect the cellular distribution of latent infection at a gross level. Next we quantified the frequency of cells that expressed either ORF73, the mRNA that encodes LANA, or any viral UMI. This analysis identified that WT LANA+ cells had a higher frequency of both ORF73 RNA+ and viral UMI+ cells compared to cycKO LANA+ cells (Fig 7K and 7L). These data suggest that even within LANA+ cells, the v-cyclin demonstrates a modest role in promoting viral gene expression in cells harvested during a well-established latent timepoint.
The v-cyclin is dispensable for reactivation in LANA expressing cells

LANA has been previously shown to play a critical role in γHV68 latency and reactivation [40], and sort purification of LANA::βla+ cells from WT infected mice enriches for cells capable of ex vivo reactivation [29], suggesting that LANA protein expression may identify reactivation-prone cells. Given the reactivation deficit observed following cycKO infection [30], and the reduced frequency of LANA::βla+ cells following cycKO infection, we postulated that the defect in reactivation of cycKO viruses observed in bulk cell explant may be a direct consequence of the reduced frequency of LANA expressing cells. To test this, we sort purified LANA::βla+ cells from mice infected with either WT,βla or cycKO,βla virus and measured reactivation capacity ex vivo. Given the low frequency of βla+ cells in healthy B6 mice (Figs 3B, 4C, and 5A), we sorted LANA::βla+ cells from CD8−/− mice, a strain of immunodeficient mice with an increased viral load where the v-cyclin is required for reactivation in bulk cell explant (discussed above). CD8−/− mice were infected with WT,βla or cycKO,βla virus via IP injection with peritoneal cells harvested at 16 dpi, stained for β-lactamase expression and FACS purified into either LANA::βla− or LANA::βla− populations (Fig 8A), followed by flow cytometric
Fig 8. The viral cyclin is dispensable for reactivation in latently infected cells that express LANA::βla. (A) Experiment schematic. CD8−/− mice were infected via IP injection with WT::βla or cycKO::βla viruses and peritoneal cells were harvested at 16 dpi. Cells were stained for β-lactamase activity and LANA::βla+ cells were sorted by FACS. (B) Gating strategy of LANA::βla sort (left) and purity of post-sort WT::βla infected LANA::βla+ cells (right). LANA::βla+ cells are located in the upper right polygon and LANA::βla− cells are located in the upper left polygon. The percent of events within each gate is indicated below the gate, with data showing that >97% of purified cells were LANA::βla+ cells. (C) WT::βla (black) and cycKO::βla (red) infected pre-sorted (left), LANA::βla− (middle), and LANA::βla+ (right) cells were subjected to limiting-dilution reactivation analysis. Reactivation was measured 21 days after plating sorted and pre-sorted cells on permissive MEFs. Linear regression with comparison of the LogEC(63.2) found that the frequency of ex vivo reactivation was higher in pre-sorted WT::βla than in cycKO::βla infected cells, whereas WT and cycKO cells had comparable reactivation when analyzed in sorted, LANA::βla+ cells. n = 2 independent experiments.
with a total of 15 WT.βla infected mice and 30 cycKO.βla infected mice. The table below lists the number of cells plated to reach CPE in 63.2% (the dotted line) of the wells plated for reactivation, corresponding to the number of cell required to find at least 1 reactivating cell.

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analysis of sort purity (Fig 8B). For each sort, we recovered ~25,000–100,000 WT.βla infected LANA::βla+ cells, ~21,000–24,000 cycKO.βla infected LANA::βla+ cells, and ~1.5×10^6 LANA::βla− cells for each virus. Bulk, LANA::βla+, and LANA::βla− cells were plated by serial dilution on MEF monolayers and assessed for reactivation 21 days post-plating. As expected, in the pre-sorted population, the cycKO.βla virus showed a reactivation defect relative to WT. βla infected peritoneal cells (Fig 8C). Note that mechanical disruption of pre-sorted population of cells resulted in no cytopathic effect on MEF monolayers, indicating that these cells did not have preformed virus and that detected virus was derived from reactivation from latency [41]. Reactivation in the LANA::βla+ population was extremely low, consistent with previous reports [29]. In contrast to the low frequencies of reactivation in LANA::βla− cells, LANA::βla+ cells demonstrated a much higher frequency of reactivation (Fig 8C). Notably, LANA::βla+ cells derived from WT.βla and cycKO.βla infected samples had equivalent reactivation frequencies (Fig 8C). These data directly demonstrate that LANA expressing cells are enriched in their ability to reactivate from latency relative to LANA negative cells, and further show that the v-cyclin is dispensable for ex vivo reactivation in LANA expressing cells. Based on these observations, the simplest explanation for the reactivation defect observed in bulk cultures from cycKO infected mice results from a defect in LANA+ latent infected cells in the cycKO infected latent reservoir. These data strongly suggest that a primary function of the v-cyclin is to promote the frequency of LANA expressing cells during latent infection in vivo, a cellular state that is competent for ex vivo reactivation from latency.

Discussion

The balance between latency and reactivation is of critical importance in γHV infection and disease progression. Chronic infection with γHV through maintenance of latency and reactivation has long been associated with virus-induced malignancies [1]. Here, we find that a primary function of the v-cyclin is to promote LANA expression during latent infection, to facilitate a reactivation competent latent reservoir. In the work presented here, we show that LANA expression is a strong correlate with reactivation capacity, while cells that fail to express LANA have limited reactivation potential (Fig 8C), consistent with published reports. These findings suggest that γHV68 latency is not a uniform state and that either the viral cyclin, or potentially host cyclins, promote reactivation by altering the “state” of the latently infected cell (Fig 9). In this specific instance, expression of v-cyclin increases the pool of LANA expressing latently infected cells, which are more permissive to reactivation from latency (Fig 9).

The value of γHV68 LANA as a marker of infected cells is due to the fact that it is expressed through all stages of infection [42]. While its requirement in latency is widely appreciated, it has also been demonstrated to be required for acute replication in vivo [42], for reactivation from latency [40,43], with context-dependent requirements for LANA that vary with infection stage and cell type [44]. Virus episome maintenance proteins, including the EBV EBNA1 and the KSHV and γHV68 LANA, are required to coordinate viral gene expression, DNA replication, and stable genome copy number [45]. Like EBV EBNA1 protein, LANA is also required for viral genome persistence and segregation to daughter cells after cell division. To achieve these functions with minimal detection by the host immune response, the expression of LANA is subject to multiple transcriptional and translational mechanisms of control [29,46]. Transcription of LANA is positively supported by LANA autoregulation and negatively regulated
Finally, the γHV68 LANA contains both conserved and divergent features in comparison to the KSHV LANA [49–51].

In the current study, we have used the γHV68 system to quantify the frequency of cells that contain viral DNA, the frequency of cells capable of ex vivo reactivation, and the cellular distribution and frequency of cells characterized by expression of LANA, a prominent latent gene in γHV68 and KSHV. Our detailed analysis of gene expression and reactivation competence in LANA+ cells focused particularly on peritoneal cells, a compartment in which B cells and macrophages are primary latently infected cell types. Whether the v-cyclin has a role in influencing plasma cell differentiation, a rare subpopulation of cells associated with reactivation, especially in lymphoid tissues, remains unanswered at this time. One limitation of these studies is that, at this time, there are features of latent infection that are less amenable to direct ex vivo analysis from primary infection (e.g. viral genome circularization, chromatinization, and tethering to the host chromosome). Future, detailed molecular characterization of primary latently infected cells may afford further insights, including determining whether all cells that contain viral DNA represent true, latently infected cells, or whether some viral DNA+ cells may reflect a dead-end infection characterized by residual viral DNA. While it is tempting to speculate that LANA::βla- cells may represent a defective state of latency, the LANA::βla reporter identifies
cells with active protein expression, and this system does not identify whether a cell may ultimately induce LANA expression (e.g. during cell division). It is interesting to note that while EBNA1 is expressed in EBV latency, and has a critical role in episome maintenance, studies of patients with infectious mononucleosis revealed that not all cells express detectable EBNA1, with EBNA1 expression found particularly in dividing cells [52]. Based on this analogy, it is possible that LANA+ cells may have enhanced reactivation-competence due to the activation or cell cycle status of the latently infected cell.

The notion that viral latency is a diverse and complex state of infection was originally defined in EBV infection, in which there are several distinct types of latency [53], with distinct reactivation potential. Further, different EBV latency programs are associated with specific clinical outcomes and pathologies [54,55]. It has also been proposed that reactivation from latency goes through a distinct intermediate step, termed animation [56], that is required for progression to virus replication and reactivation, but represents a reversible stage. While our findings likely reflect a conserved feature of biology amongst γHV's, similar trends may also be present in latent infection across virus families. For example, in HIV infection, virus persists within the host through latency in many cells types, including CD4 T cell subsets and myeloid cells [57,58]. Since these reservoirs cannot be cleared by therapeutics or the host immune system, one potential strategy for eradication of the virus is to trigger reactivation, resulting in death of the cell by the virus or the host immune system [59]. One barrier to this approach is the fact that distinct populations of latently infected cells appeared differentially responsive to reactivation stimuli [60].

This study provides the first evidence that the requirement for the viral cyclin in γHV68 reactivation is linked to LANA expression during latency, a reactivation-prone cellular phenotype. This conclusion is based on the observation that LANA+ cells are equivalent in reactivation frequency when comparing WT and cycKO LANA+ cells. Further, single cell transcriptomic analysis of WT and cycKO LANA+ cells demonstrates strong similarities in gene expression. Important questions are raised by these findings, including whether the v-cyclin promotes LANA expression through a transcriptional, translational, and/or post-translational mechanism, and whether the v-cyclin promotes expression of additional latency associated genes. For example, the v-cyclin may enhance the positive feedback loop mediated by LANA autoregulation or the v-cyclin may antagonize p53 function, thereby enhancing LANA transcription. It is also possible that the v-cyclin may facilitate optimal epigenetic regulation of the viral genome, to promote a reactivation-prone state. While the v-cyclin promotes the frequency of LANA expressing cells, it is notable that latent LANA expression can occur in a v-cyclin independent manner (i.e. LANA+ cells in cycKO infected mice). In our previous studies, we have identified two contexts in which reactivation can occur in a v-cyclin independent manner. First, host cyclin D3 is capable of fulfilling the role of v-cyclin in driving reactivation in a cycKO background, albeit with a decreased efficiency [61]. Second, genetic, or physiological, loss of p18Ink4c enables robust reactivation of γHV68 in cycKO infection, comparable to WT infection [27,28]. In this latter study, we also found that the frequency of LANA+ cells was comparable between WT and cycKO infection in p18-deficient mice, again suggesting a link between the LANA+ phenotype and ex vivo reactivation-competence [28]. Based on these observations, it is possible that cycKO infected cells with LANA expression may reflect cells with either increased cellular D-type cyclin expression and/or decreased p18Ink4c expression, facilitating LANA expression and reactivation. Although our data emphasize that the v-cyclin promotes LANA expression and that LANA expressing cells are reactivation competent, it remains to be tested whether the v-cyclin supports additional features for optimal reactivation capacity. Finally, we also previously demonstrated that, in the absence of B lymphocytes, the
viral cyclin is required for long-term maintenance of latent infection [33], a role that may also be due to this newly appreciated role of the cyclin in supporting LANA expression.

The work described here documents a critical link between v-cyclin and viral LANA expression in reactivation from latency. Further, our findings suggest that the latently infected reservoir, defined by the frequency of cells containing viral genome during latency, is characterized by at least two stages that vary in gene expression and reactivation capacity, and whose balance is regulated by the v-cyclin. These data strongly suggest a previously undescribed v-cyclin/LANA axis that is critical for reactivation from latency and emphasize that efforts to manipulate this axis may require a combinatorial approach that targets both v-cyclin dependent and independent processes to effectively disrupt the latent reservoir.

Methods and materials

Ethics statement

The animal study 00189 was reviewed and approved by the Institutional Animal Care and Use Committee according to the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Cell lines and viruses

3T12 mouse fibroblast cells (ATCC CCL-164) were cultured in 5% FBS/DMEM with 20 units of penicillin and 20 μg of streptomycin per mL and 4 mM L-glutamine. MEFs were isolated as described and cultured in 10% FBS/DMEM with 20 units of penicillin per mL, 20 μg of streptomycin per mL, 4 mM L-glutamine, and fungizone at 250 ng/mL [41]. Generation of the WT. βla and cycKO.βla viruses has been previously described [28,29,32].

Mice

All animal work was performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, Animal Welfare Assurance of Compliance: # D16-00171 and approved by the CU Institutional Animal Care and Use Committee #00189. C57BL/6 (B6) mice were obtained from the Jackson Laboratory (Stock # 000664). CD8α−/− mice on the B6 background (CD8−/−) were obtained from the Jackson Laboratory (Stock # 002665) and have been previously described [39]. CD8−/− mice were bred in house at the University of Colorado Denver Anschutz Medical Campus in accordance with University regulations and Institutional Animal Care and Use Committee.

Flow cytometry analysis

Spleens were collected and splenocytes were isolated in a single cell suspension after being passed through a 100 micron filter. Splenocytes were then subjected to red blood cell lysis by treatment with red blood cell lysis buffer (Sigma # R7757) per manufacturer’s recommendation. Peritoneal cells were collected with 10 mLs of cold 1% FBS DMEM. β-lactamase activity was detected using the LiveBLAzer FRET-BG/Loading Kit with CCF2-AM (ThermoFischer Scientific # K1025) as previously described [17,29,32]. Cell surface antibodies used were CD19-AlexaFluor 700 (clone eBioID3, eBioscience # 56-0193-81), CD38-APC (clone 90,eBiosciences #17-0382-81), IgD-APC-Cy7 (clone 11-26c.2a, Biolegend # 405716), and CD5-APC (clone 53–7.3, eBioscience # 17-0051-81). Fc blocking antibody 24G2 was used in staining to prevent antibody binding to cellular Fc receptors.
Limiting-dilution analysis
Mice were inoculated with either WT.βla or cycKO.βla at 1x10^6 PFU/mouse via IP injection. After 8 and 16 days, splenocytes and peritoneal cells were collected as above and analyzed by either flow cytometry or plated for reactivation or PCR analysis. By Poisson distribution, the number of cells plated corresponding to 63.2% of the wells positive is the frequency at which there is at least one reactivating or genome positive cell, respectively.

Reactivation analysis. Cells were subjected to serial limiting dilution analysis, and plated on highly permissive MEF monolayers for quantification of virus cytopathic effect as previously described [12,33]. To control for any preformed virus, mechanically disrupted peritoneal cells were plated in parallel; no monolayer disruption was observed in disrupted cells.

LD-PCR analysis. Cell dilutions were subjected to in-plate DNA isolation and nested-PCR for single copy sensitivity detection of viral gene 50 DNA, with plasmid sensitivity controls included on each plate, as previously described [12,33].

Quantitative-PCR analysis
CD8/- mice were infected with 1x10^6 PFU of either WT.βla (n = 3) or cycKO.βla (n = 3) virus or mock infected (n = 2) via IP injection. At 16 dpi, peritoneal cells from individual mice were harvested from each mouse, pelleted at 1,000xg for 10 min, resuspended in RLT buffer containing β-mercaptoethanol and then frozen at -80°C. Cells were then thawed and homogenized via Qiashredder columns and RNA was isolated using the RNeasy Micro Kit. DNA was removed from the samples by treating with Turbo DNase as per the manufacturer’s recommendations (ThermoFisher). cDNA was synthesized using SuperScript II Reverse Transcriptase (ThermoFisher). Primers for SYBR Green qPCR were designed using Primer3. Primers used were: LANA Forward 5'-ATCAGGGAATGCGAAGAC AC, LANA Reverse 5'- GTGCTGTGGTACCAAGGGTAA, β-lactamase Forward 5’-GCTATGTGGCGGTTATTTAT, β-lactamase Reverse 5’- AAGTTGGCCGCAGT GTTATC. iQ SYBR Green Supermix was used for the qPCR reactions (Bio-Rad) and qPCR was performed with technical triplicates from the peritoneal cell cDNA of each mouse, and run on the QuantStudio 7 Flex instrument.

Single cell transcriptome analysis
CD8/- mice were inoculated with 1x10^6 PFU of either WT.βla (10 mice) or cycKO.βla (10 mice) or WT γHV68 (2 mice) viruses or mock inoculated (n = 2) via IP injection. At 16 dpi, peritoneal cells were collected and combined for each virus group, and stained for β-lactamase. These cells were then sorted by the Clinical Immunology Flow Core with the University of Colorado Anschutz Medical Campus. Mock and WT γHV68 infected peritoneal cells were used to identify background fluorescence. WT.βla and cycKO.βla infected PerC were sorted for live βla+ cells, resulting in 60,534 WT.βla and 25,172 cycKO.βla cells collected, with 3000 cells from each loaded for capture by 10x Chromium single cell RNA sequencing.

FACS sorted reactivation
CD8/- mice were infected with 1x10^6 PFU of either WT.βla or cycKO.βla virus via IP injection. At 16 dpi, peritoneal cells were collected and combined for each virus group. For each virus group, 1x10^6 cells were set aside as “pre-sorted” cells. The remaining cells were stained for β-lactamase then washed and resuspended in 2% FBS in PBS. These cells were then sorted by the Clinical Immunology Flow Core with the University of Colorado Anschutz Medical Campus. Cells were gated as single cells and then sorted into βla+ or βla- populations. A small number of LANA::βla+ WT.βla infected cells were tested for purity after the sort had
concluded. The purity of the LANA::βla+ cells was measured in the WT.βla infected samples and found to be 97.3% pure. A corresponding purity check was not performed for the cycKO.βla infected samples due to a lower total number of cells recovered. Pre-sorted, LANA::βla+, or LANA::βla− cells were diluted into 10% FBS in DMEM and plated onto permissive MEFs in a limiting-dilution fashion as previously described [12,33]. Pre-sorted and LANA::βla− cells were plated at starting concentrations of 2x10^4 cells per well while LANA::βla+ cells were plated at a starting concentration of 100 cells per well. Three weeks after plating cells, reactivation was measured by observation of cytopathic effect on the MEF cells.

**Statistical analysis and software**

Flow cytometric analysis was performed using FlowJo V.10.0.8r1. Graphs were generated and statistical analysis were performed using GraphPad Prism 7.0a. Limiting-dilution curves were created by performing a non-linear regression, log(agonist) vs. response- using the “EC anything” regression equation where F was set to 63.2, with top and bottom of the curves constrained to 100 and 0 respectively. Comparisons of the LogECF were used to determine statistical significance. Unpaired student t-tests were performed as mentioned. Quantitative-PCR data was analyzed using the Pfaffl method [62] and graphed using GraphPad Prism 7.

**Supporting information**

S1 Fig. Disruption of the viral cyclin results in a reduced frequency of LANA::βla+ cells in the lungs of C57BL/6j mice after intranasal infection. Mice were infected via I.N. inoculation with WT.βla or cycKO.βla viruses and lung were harvested at 8 dpi. (A) Representative pseudocolor plots identifying βla+ lung cells in the upper right polygon. Frequency of βla+ cells is indicated below the gate +/- SEM. (B) The percent of lung cells that are βla+ for each mouse is plotted with SEM shown after infection with WT.βla (black) or cycKO.βla (red). (C) The total number of βla+ cells per lung for each mouse is plotted with SEM shown after infection with WT.βla (black) or cycKO.βla (red). WT.βla n = 6 cycKO.βla n = 7. Two-tailed student t test was used for statistical analysis.

(TIF)

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