Kaposi’s sarcoma-associated herpesvirus (KSHV) is a human pathogen that establish latent infections in the host. The protein responsible for establishment of latency is the latency-associated nuclear antigen (LANA). Although, one of the biggest problems to the study these herpesviruses are their narrow host tropism, the identification of murid herpesvirus 68 (MHV-68), a mouse equivalent virus which also encodes a LANA protein (mLANA), enabled the possibility of development of a mouse model to study KSHV pathogenesis. Previous studies showed that the replacement of mLANA by kLANA in a MHV-68 background was a good model to study KSHV pathogenesis in a mouse model of infection. The aim of this work was to test the influence of the aspartate and glutamate (DE) repeat region, in an *in vivo* infection, by generating a recombinant virus: v-kLANA Δ331-495, in which the DE region was deleted. Two other recombinant viruses from previous work were added to this study, in order to complement the results: v-kLANA Δ465-929 and v-kLANA Δ332-929. *In vivo* results showed that v-kLANA Δ331-495 establish very low levels of latency in the spleen, with values lower than v-kLANA and v-kLANA Δ465-929, but higher than v-kLANA Δ332-929. Altogether, these data indicate that within the internal region of LANA, the DE region must exert a more important role in the establishment of latency in the spleen, during an infection.

**Keywords:** KSHV; LANA; MHV-68; internal repeats; latency; *in vivo*.

Kaposi’s sarcoma-associated herpesvirus (or human herpesvirus 8) is a gamma-2-herpesvirus that was first identified in 1994 by Yuan Chang and his colleagues (Chang *et al.*, 1994). It is the etiological agent of Kaposi’s sarcoma (KS), primary effusion lymphoma (PEL) and multicentric Castleman’s disease (MCD) (Coscøy, 2007; Wu *et al.*, 2010).

The genome of KSHV consists in a long unique region (LUR) with approximately 145 kb which is flanked by 20-35 kb of non-coding terminal repeats region with a high G+C content (Juillard *et al.*, 2016; Moore and Chang, 2003). KSHV life cycle has two distinct phases of infection: a lytic infection and a persistent latent infection, both with different patterns of gene expression and both contribute to the pathogenicity of the virus (Uppal *et al.*, 2014; Wu *et al.*, 2010). In latently infected cells, viral genome is maintained as a multi-copy, non-integrated episome (Wen and Damania, 2010; Wu *et al.*, 2010).

KSHV latency-associated nuclear antigen (kLANA) is the responsible protein for the establishment of latency and it is constituted by a proline-rich N-terminal domain, an internal repeat region and the C-terminal DNA binding domain (DBD) (Figure 1A) (Ponnusamy *et al.*, 2015). There are two key components to episome persistence in proliferating cells and kLANA is responsible for both: the episomal DNA need to replicate with each cell division and the epimises have to segregate to daughter cells nuclei following mitosis to avoid their destruction in the cytoplasm (Juillard *et al.*, 2016).

Only *in vitro* studies were performed to try to understand a possible function for internal repeat region, which is the more uncharacterized part of kLANA. This internal region is constituted by three segments: an aspartate and glutamate (DE) region, from amino acid 330 to 430, a glutamine (Q) region, from amino acid 440 to 756 and a leucine (L), valine (V), glutamate and glutamine...
(EQE) spaced in a leucine-zipper-like pattern (LZ), from amino acids 760 to 931 (Figure 1A) (Alkharasah and Schulz, 2012). In 2011, De Leon Vasquez and Kaye fused N- and C-terminal of LANA deleting all the internal repeat region and observed that mutants were highly deficient in episome maintenance (De Leon Vasquez and Kaye, 2011). In 2013, they constructed a series of LANA mutants with different deletions and results suggested that the internal repeat region between amino acids 331 to 465 may have an important contribution to episome replication (De Leon Vasquez et al., 2013).

One of the biggest problems to the study of human herpesvirus is their narrow host tropism, so the identification of murid herpesvirus 68 (MHV-68), a mouse equivalent virus, which also encodes a LANA (mLANA), enabled the possibility of development of a mouse model to study gammaherpesvirus pathogenesis (Simas and Efstathiou, 1998). mLANA and kLANA are homologous, so Habison et al. engineered a recombinant virus where mLANA was replaced by kLANA in MHV-68 genome and showed that this is a viable model to study KSHV pathogenesis in vivo (Correia et al., 2013; Grundhoff and Ganem, 2003; Habison et al., 2017).

The aim of this project was to assess the impact of kLANA DE internal repeat in the establishment of latency after an in vivo infection. To do this assessment, a deletion between amino acids 331-495 of kLANA (v-kLANAΔ331-495) was performed (Figure 1A) and introduced in MHV-68 genome. Results showed that v-kLANA Δ331-495 establish very low levels of latency in the spleen, with values remarkably lower than v-kLANA.

Materials and Methods

Cell lines. Baby hamster kidney (BHK-21) fibroblasts were maintained in Glasgow Minimum Essential Medium (GMEM) supplemented with 10% fetal bovine serum (FBS), 10% tryptose phosphate broth (TPB), 100 U/mL penicillin-streptomycin and 2mM L-glutamine. Mouse embryonic fibroblasts (NIH-3T3) expressing the CRE recombinase were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented as above, except TPB.

Plasmids and cloning. The fragment between the PstI restriction site in the 5’ UTR of kLANA and codon for amino acid 330 of kLANA was amplified by PCR, using pSP72_PCR1_4 (contains DNA encoding the full length kLANA (KSHV genome coordinates 123808-127886) and its 5’ untranslated region (UTR) flanked at the left side by the MHV-68 upper flank (genome coordinates 104710-105092)) as a template and using primers specific for this amplification: IMMAF3 (Forward) 5’-GTACTGACGGCTGCTAGTG-3’ and IMMAF2 (Reverse) 5’-AAGCTGACATCTTTAGCTTCAGCTC-3’. Then, amplified fragment was purified and cloned into pSP72_PCR1_4 using the PstI restriction sites (underlined in the primer sequences). Resulting plasmid was pSP72_PCR1_4 kLANA Δ331-495. After that, using Xhol and HindIII restriction enzymes MHV-68 lower flank from pSP72_PCR1_5 (similar to previous plasmid, but contains also MHV-68 lower flank with a restriction site of BglII) was subcloned into pSP72_PCR1_4. The resulting plasmid was pSP72_kLANA Δ331-495. Finally, pSP72_kLANA Δ331-495 was digested with BglII and subcloned into BamHI-G shuttle plasmid (it contains the MHV-68 genomic BamHI-G fragment (genome coordinates 101,654-106,903, U97553) cloned in pST76K-SR (shuttle). The BamHI-G fragment contains the mLANA coding sequence (genome coordinates 103,927-104,868)).

Generation of recombinant viruses. MHV-68 recombinant virus was generated by a mutagenesis procedure in E. coli DH10B, according to the Two-Step-Replacement Strategy, described by O’Connor et al. (O’Connor et al., 1989). BamHI-G shuttle containing the kLANA with the desired mutations was transformed into E. coli DH10B competent bacteria harboring a BAC with the entire WT MHV-68 genome cloned, using the heat shock method. Bacteria were spread in LB plates containing the selection marker of shuttle plasmid (kanamycin) and the selection marker of BAC (chloramphenicol) and incubated 1-2 days at 30°C. In this step, a recombination event between homologous MHV-68 sequences in the BamHI-G recombinant shuttle and MHV-68 BAC. This protein is encoded by the shuttle plasmid and it is responsible for the complete integration of BamHI-G shuttle plasmid into BAC genome, forming the co-integrates. To select these co-integrates, the bacteria colonies were subsequently spread in LB plates containing kanamycin/chloramphenicol and incubated overnight at 43°C. This step was repeated to ensure that the co-integrates are picked. Afterwards, the largest colonies were picked and...
plated only on LB agar with chloramphenicol and incubated 1-2 days at 30°C. In order to isolate the resolved clones with the BAC mutants, the bacteria were spread into LB agar with chloramphenicol and 5% of sucrose and incubated 1-2 days at 30°C. After, the colonies were picked once and plated in parallel first in kanamycin and then in chloramphenicol LB agar, and incubated overnight at 37°C. To identify MHV-68 BAC recombinants containing kLANA coding sequences, a colony PCR was performed using primers that hybridize to the 3’end of kLANA: upper primer - 1019 F (5’-GTCCTTACAGAGATGATGATTG-3’) and lower primer - 1150 R (5’-AAAAGCTTATTTCCCTGGCCTGGGTTAATG-3’). PCR positive clones were grown in LB medium with chloramphenicol and minipreps were prepared and characterized by restriction profile analysis using EcoRI and BamHI to assess the integrity of the genome and confirm kLANA desired deletions. Clones with the expected profile were grown and BAC maxipreps were prepared. Viruses were reconstituted by transfecting BAC DNA into BHK-21 cells using X-tremeGENE HP (RocheAppliedScience). The loxP-flanked BAC cassette was removed by viral passage through NIH-3T3-Cre cells.

**Viruses.** The wild type virus (v-WT) used in this work was derived from a genomic BAC and it is essentially a MHV-68 clone G2.4, that was isolated from virus grown in BHK-21 cells, but contains a single loxP site (Efstathiou et al., 1990; Adler et al., 2000; Adler et al., 2001). MHV-68 recombinant virus expressing yellow fluorescent protein (yfp) was driven by the human cytomegalovirus (HCMV) and cloned in the intergenic region between open reading frames 27 and 29b (Collins et al., 2009). Chimeric kLANA MHV-68 virus, in which the mLANA was replaced by the full length kLANA and 5’ UTR (v-kLANA). The chimeric kLANA MHV-68 expressing yfp was constructed in Psimas Lab (Habison et al., 2017). v-kLANA Δ465-929 is a recombinant virus that has a deletion encompassing amino acids 465 to 929, which removes part of the glutamine region (Q), all the leucine zipper (LZ) and the glutamate and glutamine (EQE) regions. v-kLANA Δ332-929 is a recombinant virus that has a deletion between amino acids 332 to 929, having the totality of the internal repeat region of kLANA deleted.

**Viral stocks and titration.** Working viral stocks were produced by infecting 5x10^6 BHK-21 cells with a MOI of 0.002 plaque forming units (PFU)/cell, in 175 cm^2 flasks. Cells were incubated for 4 days at 37°C, 5% CO_2 and after this period, they were scrapped into the media, centrifuged for 5 min at 4°C at 1500 rpm. Supernatant was transferred to 30 mL bottles and ultracentrifuged at 12000 rpm, during 2h at 4°C to pellet virus. Pellet was added 2mL of fresh media (softened for 1h). Pellet was resuspended in that same volume, distributed in 75µL aliquots and stored at -80°C (working stock media (WSM)). The cell pellet was resuspended in 2mL of fresh supplemented GMEM, aliquoted and stored at -80°C (cell working stocks (CWS)).

For virus titration, 1mL of 2.5x10^5 BHK-21 cells were incubated in a rotating table with viral 10-fold serial dilutions (10^-3 to 10^-8) for 1h at RT in 15 mL falcon tubes. After this period, 2mL of fresh supplemented GMEM were added to the tubes and cells/virus mixture was added to 6-well plates. Plates were incubated for 4 days at 37°C. Cells were fixed with 4% formaldehyde in PBS and stained with 0.1% toluidine blue. Viral plaques were counted using a magnifying glass. Titer was given using the formula:

\[ \text{Titer} = \frac{\text{nr of plaques}}{\text{dilution}} \times \frac{1}{\text{inoculum}} \]

**Multi-step growth curve.** 5x10^4 BHK-21 cells/well were seeded in 24-well plates and infected with 0.01 PFU/cell in 200µL of complete GMEM. Cells were incubated during 1h at 37°C, Inoculum was removed, cells were washed twice with 500µL of PBS, 1mL of fresh media was added in each well and cells were incubated at 37°C. Cells and media were recovered at 0, 24, 48, 72, 96 and 120 hours post-infection (h.p.i.) and frozen at -80°C. Virus titers were determined by plaque assay in duplicate.

**Immunoblotting.** BHK-21 cells were infected with 3 PFU/cell for a total of 6h. Cells were washed with PBS and lysed with lysis buffer (150mM NaCl, 10mM Tris-HCl pH 7.4, 1mM Na3VO4, 1mM NaF, 1% TritonTM X-100, complete protease inhibitors, MilliQ H2O). Lysates were centrifuged and 1.25x10^5 cells equivalents were loaded in a 10% SDS-PAGE gel. Proteins were detected with rat α-kLANA LN53 1:1000 (Advanced Biotechnologies), mouse α-mLANA mAb 6A3 1:10 (Pires de Miranda et al., 2012), rabbit α-M3 1:2000 (Jensen et al., 2003), mouse α-EGFP 1:2000 (Sigma) and rabbit α-Actin 1:2000 (Sigma). Secondary horse...
radish peroxidase conjugated antibodies were from Jackson ImmunoResearch.

**In vivo assays:**

**Infection of mice.** 7-week-old female C57BL/6 J purchased from Charles Rivers Laboratories were anaesthetized with isoflurane and infected intranasally with 10⁴ PFU of virus in 20µL of PBS. At 14 days post-infection, mice were sacrificed by CO₂ inhalation and the spleens were removed to a falcon containing 5mL of PBS+2% FBS.

**Single cell suspensions.** Spleens were mechanically disrupted and filtered through a 100µm cell strainer, in order to remove some debris. Cells were centrifuged at 1200 rpm, for 10 min at 4ºC, and the pellet was resuspended in 1mL of Red Blood Cell Lysis Buffer (RBL) (154mM ammonium chloride, 14mM sodium hydrogen carbonate, 1mM EDTA pH 7.3). Cells were incubated on ice for 5 min and then, washed with 10µL of PBS+2% FBS. Cells were centrifuged at 1200 rpm, for 5 min at 4ºC. Pellet was resuspended in 1mL of PBS+2% FBS and each splenocyte suspension was divided in two falcons (500µL each): one falcon was used to assay viral latency by infectious center assay/reactivation assay (ICA) and the other falcon was used to flow cytometry analysis.

**Infectious center assay.** Latent virus titers were determined by a reactivation assay. Here, co-culture of single cell suspension of splenocytes with BHK-21 cells, leads to reactivation of latent virus and formation of viral plaques. 10-fold serial dilutions of splenocyte suspension were prepared and plated, in duplicate, in 6 cm² plates containing 4.5x10⁶ BHK-21 cells. Plates were incubated for 5 days at 37°C. Spleens were also analyzed for the presence of pre-formed infectious viruses, by performed plaque assay in freeze-thawed splenocyte suspensions. Plates were incubated for 4 days, at 37°C. In both assays, after the incubation period, cells were fixed with 1% formaldehyde in PBS and stained with 0.1% toluidine blue. Viral plaques were counted in a magnifying glass and infectious centers (PFU/spleen) were determined.

**Fluorescent-activated cell sorting.** Splenocytes were filtered through a 40µL cell strainer and incubated with purified Rat anti-mouse CD19/CD32 (1:100 in PBS+2% FBS) for 15 min on ice, to block Fc receptors. After washing with PBS+2% FBS and centrifugation, splenocytes were surfaced stained by incubation for 25 min on ice, in the dark, with the appropriated antibodies diluted in PBS+2% FBS: APC-H7 Rat anti-mouse CD19 (1:400) (BD Biosciences), Anti-Human/Mouse GL7 eFluor® 660 (1:200) (ebiosciences) and PE conjugated hamster anti-mouse CD95 (1:800) (BD Biosciences). Later, cells were washed twice with PBS+2% FBS, to remove the unbound antibodies and centrifugation. Cells were resuspended in PBS+2% FBS and transferred to FACS tubes. For each infection group, 1x10⁸ GC B cells (CD19⁺CD95⁺GL7⁺) were purified by a BD FACSAria (BD Biosciences) cell sorters. The total splenocytes were analyzed on a LSR Fortessa (BD Biosciences), using FACSDiva software (BD Biosciences) for acquisition and FlowJo (Tree Star, Inc.) for analysis.

**Limiting dilution and real-time PCR of viral DNA-positive cells.** A pool for each infectious group was prepared (5 mice per pool), from the splenocyte suspensions. Splenocytes were diluted to 2x10⁶ cells in 100µL PBS+2% FBS. 2-fold serial dilutions were prepared and lysated with lysis buffer (10mM Tris·HCl pH8.3, 3mM MgCl₂, 50mM KCl, 0.45% NP-40, 0.45% Tween-20, 0.5mg/mL proteinase K). PCR tubes were left overnight at 37°C and proteinase K was inactivated at 95°C for 5 min. Eight replicates of each dilution were analyzed by real-time PCR, on a Rotor Gene 6000 thermocycler (Corbett Life Science) using a fluorescent Taqman probe and primers specific for the MHV-68 M9 gene: M9-F (upper primer): 5'- GCCACCGGTGCGGCTCTA-3’; M9-R (lower primer): 5'- CAGGCCCTCCCTCCTTG-3’ and M9-T probe: 5’- 6-FAM - CTT CTG TTG ATC TTC C – MGB-3’. PCR reactions was prepared in a total volume of 25µL, containing 2.5µL of cell suspension lysate, 200µM of each primer, 300µM of probe, 1x Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 5mM MgCl₂ and nuclease free water. The cycling program consisted of an initial melting step of 95°C for 10 min followed by 40 cycles of amplification, starting at 95°C for 15 sec and then 60°C for 1 min. In all PCR runs, a positive control was added. This positive control consisted of a series of dilutions of pGBT9-M9, a plasmid that contains the M9 gene. Real-time PCR results were analyzed on the Rotor Gene 6000 software. In all dilutions tested each replicate was scored positive or negative based on comparison with the negative (water sample) or the positive (plasmid containing M9 gene) controls.
Statistical analysis. All the statistical analysis was performed with GraphPad Prism Software. To calculate the frequency of cells with viral DNA, for limiting dilution analysis, 95% confidence intervals were determined as described (Marques et al., 2003).

Results

Generation and characterization of MHV-68 recombinant virus harboring a mutation on kLANA.

As described above, mLANA and kLANA share a lot of similar characteristics. Habison et al. replaced the endogenous ORF73 (mLANA) of MHV-68 genome by KSHV ORF73 (kLANA) and 5′UTR, but not the kLANA promoter, to assess the viability of this chimeric virus to study kLANA in vivo. The resulting chimeric virus was named v-kLANA. They showed that the levels of latency in vivo of v-kLANA were lower compared to the v-WT, but both v-WT and v-kLANA infected cells had similar genome copy numbers. Thus, kLANA rescued mLANA deficient MHV-68, allowing the chimeric virus to establish a latent infection in vivo (Habison et al., 2017). This finding shows that this is a viable model to study KSHV pathogenesis in vivo by infect small animal models with v-kLANA.

As the internal region of kLANA is constituted by a series of internal repeats and their functions are not fully known, some studies in vitro are being done to address the relevance of the different kLANA internal repeats. The aim of this work was to evaluate the importance of kLANA DE internal repeats in vivo, so a recombinant virus containing a deletion encompassing amino acids 331 to 495 of kLANA, which corresponds to all the DE internal repeat region, was engineered (v-kLANA Δ331-495) in backgrounds of wild type MHV-68 (non-yfp) or yellow fluorescent protein (yfp). However, two other recombinant viruses from previous work in our laboratory were added to the in vitro and in vivo experiments, in order to complement the study (unpublished data, Seixas, Miranda e Simas): v-kLANA Δ332-929 and v-kLANA Δ465-929 (Figure 1A).

To confirm that after the mutagenesis procedure the mutant BAC’s maintained their integrity, they were characterized by PCR and by restriction enzyme analysis, using EcoRI and BamHI. Results showed that there were no unexpected changes in the restriction profiles.

Expression of kLANA mutant proteins

To investigate if all recombinant viruses expressed correctly the different kLANA mutant proteins, BHK-21 cells were infected with a MOI of 3 PFU/cell during 6h and an immunoblotting was performed using antibodies against kLANA and other cellular proteins. kLANA LN53 is a commercial antibody that recognizes the repetitive glutamic motifs EQEQE found in the glutamate and glutamine repeat region (EQE) of kLANA. This antibody detected full length kLANA with a molecular weight of approximately 250 kDa and the mutant kLANA proteins with a lower molecular weight, as expected. The immunoblot worked very well, however, recombinant virus v-kLANA Δ465-929 #32 lacks the EQE internal repeat of the protein, so the detection in this mutant did not work. mLANA was detected only in v-WT sample, as expected. Detection of M3 showed that the levels of infection were similar between v-WT and chimeric viruses, indicating comparable infection levels, in the yfp background (Figure 1A). In the virus in a non yfp background, the levels in kLANA and in mutant kLANA were higher than in the WT (Figure 1B). Actin protein was also detected and worked as a control for cell infection. Levels of detection between all samples were similar. Detection levels of GFP were similar between samples, except for mutant v-kLANA Δ331-495 #27 where the detection level was lower.

In vitro growth of the recombinant viruses

To assess the growth of mutant viruses, a multi-step growth curve was performed. BHK-21 cells were infected with a MOI of 0.01 PFU/cell and every day, during 5 days, time points were collected. Later, time points were titrated by freeze-thawed, titers were determine by plaque assay and a growth curve was constructed (Figure 1C). In both assays (yfp and non-yfp) viruses had a similar growth between them and similar to the growth of other v-kLANA viruses previously observed in our laboratory (Habison et al., 2017).

In vivo assays:

Infectious center assay

To assess if recombinant viruses with the lacking parts of the internal repeat region could also establish latency in spleens, C57BL/6 J mice were intranasally inoculated with 10^4 PFU of v-WT or with recombinant viruses. Latent load in spleen (Figure 2A, closed circles) was determined by
quantification of ex vivo reactivation of competent viruses in total splenocytes by an infectious center assay at 14 days post-infection (d.p.i.). When latent viruses in the splenocytes are co-cultured with fibroblasts, they form viral plaques within cell monolayer that can be quantified. To verify if no lytic infectious viruses were present in splenocytes, the splenocyte suspensions were freeze-thawed and co-cultured with fibroblasts (Figure 2A, open circles). It was performed two assays: one with recombinant viruses in a yfp background and another with recombinant viruses in a non-yfp background.

In the yfp assay, v-kLANA.yfp was able to establish latency in the spleen with lower levels (~2 log) than those of v-WT.yfp, as previously observed (Habison et al., 2017). Levels of latency in v-kLANA Δ465-929 #32.yfp were similar to the levels of v-kLANA.yfp, as observed in previous work in our laboratory. In both clones of v-kLANA Δ331-495.yfp the levels of latency were low, compared with v-kLANA.

In the non-yfp assay (Figure 2A) the levels of latency of v-kLANA were lower (~2 log) than those of v-WT, as in the yfp assay. The latency levels of v-kLANA Δ332-929 #2 were below the detection limit of this assay and the levels of v-kLANA Δ331-495 #15 were similar to the ones observed in yfp assay. However, latency levels of v-kLANA Δ465-929 #16 were too low compared with the ones in yfp assay and with results from previous work done in our laboratory using the same virus.

Fluorescence-activated cell sorting (FACS)

Simultaneous to infection center assay, the spleens infected with v-WT or recombinant yfp viruses were analyzed by flow cytometry. Using yfp expression, it was possible to determine the frequency of GC B cells that were infected with viruses. Here, the percentage of infection varied between ~0-4%, where the higher percentage of yfp+ GC B cells was in v-WT.yfp infected mice (Figure 2B). The percentage of infected cells was almost null in v-kLANA Δ331-495 #27.yfp, which is in agreement with previous results from infectious center assay. The percentage of infected cells was unexpectedly too low in v-kLANA Δ465-929 #32.yfp, since in Figure 1A its titer was similar to v-kLANA.

Quantification of the frequency of viral DNA-positive total splenocytes

To complement the results from infectious center assay, it was also investigated the frequency of infected cells by limiting dilution coupled with real-time PCR in total splenocytes. This experiment allows to directly indicate the numbers of latently infected cells at 14 d.p.i., in spleen of mice intranasally infected with v-WT or recombinant viruses. Total splenocytes were subjected to 2-fold dilutions, with 8 replicates and lysed. Cell lysates were analyzed by real-time PCR, using primers and probe specific for MHV-68 M9 gene. The experiment with the v-WT and recombinant viruses in a yfp background did not work, so the graphic represented is only from the experiment with the viruses in a non-yfp background.

The v-WT had the expected frequency of latently infected cells in total splenocytes population (Figure 2C) and the frequency of viral DNA-positive cells for v-kLANA was ~1 log lower than v-WT (Habison et al., 2017). v-kLANA Δ331-495 #15 had a frequency of latently infected cells 2 to ~2 1/2 log lower than those of v-WT. The v-kLANA Δ332-929 #2 had a frequency of viral DNA-positive cells lower in total splenocytes compared with the other viruses, however, this result is in agreement with the results from the infectious center assay. These results are in agreement with the results from infectious center assay, except for the recombinant virus v-kLANA Δ465-929 #16 that in this experiment had a frequency of latently infected cells similar to v-kLANA, but in infectious center assay had a titer much lower than v-kLANA.

Discussion

This work led to the construction of a new v-kLANA MHV-68 recombinant virus, v-kLANA Δ331-495, containing a deletion corresponding to amino acids 331 to 495 of kLANA which encompasses the DE internal repeat region. Two other recombinant viruses from previous work (unpublished data, Seixas, Miranda e Simas) were added to this study to complement the results and compare the contributions of the DE region with other internal repeat regions: v-kLANA Δ465-929 and v-kLANA Δ332-929. Results indicate that from all the internal repeats of kLANA, the DE region is essential for an efficient establishment of latency in the spleen.
Figure 1: (A) Schematic diagram of KSHV LANA and deletion mutants. The different domains of the protein are indicated, in bold, namely proline-rich region (P), the aspartate and glutamate (DE), the glutamine (Q), the glutamate and glutamine (EQE), the leucine zipper (LZ) regions and the DNA binding domain (DBD). Amino acid residues are indicated below the different domains (adapted from De Leon Vazquez et al., 2013). (B) Detection of the expression of viral proteins in v-WT and chimeric viruses in yfp and non-yfp backgrounds, respectively. BHK-21 were infected with 3 PFU/cell during a period of 6h. The proteins were detected with the antibodies indicated on the right and the molecular weight protein markers are indicated on the left. (C) Growth curves of v-WT and chimeric viruses in yfp and non-yfp backgrounds, respectively. To assess growth of the viruses in vitro, BHK-21 were infected with 0.01 PFU/cell of the indicated viruses. At the indicated times post-infection, the samples were harvested and titrated by plaque assay of frozen-thawed samples. There was no significant difference between infectious groups.
Results of *In vitro* experiments showed that despite the mutation on kLANA, recombinant virus v-kLANA Δ331-495 expressed correctly mutant kLANA with a lower molecular weight than original kLANA, as well as other cellular proteins tested (Figure 1B). This result means that the deletion introduced did not affect expression of kLANA.

This recombinant virus has an advantage over v-kLANA Δ465-929 and v-kLANA Δ332-929, which is the fact that expression of kLANA can be detected using a very sensitive commercial antibody (LN53) that detects several epitopes in the EQE repeat region. Since v-kLANA Δ465-929 and v-kLANA Δ332-929 lack this internal repeat, expression of kLANA could not be detected.

v-kLANA Δ331-495 had a normal *in vitro* growth, similar to v-WT and v-kLANA recombinant viruses, in both non-yfp and yfp backgrounds (Figure 1C). These results were not unexpected, since it is known that ORF73 from MHV-68 is not essential for growth of the virus *in vitro* (Habison et al., 2017).
Our in vivo results from the infectious center assay showed for the first time that at 14 d.p.i v-kLANA Δ331-495 was able to establish latency in spleen with values near to the limit of detection of the assay. This recombinant virus exhibited ~1 log of deficiency compared with v-kLANA and ~3 log of deficiency compared to v-WT (Figure 2A). v-kLANA Δ465-929 had a peak of latency similar to v-kLANA, which is in agreement with results from previous work in our laboratory. v-kLANA Δ332-929 failed to establish latency, presenting levels below the limit of detection of this assay. These results showed that the capacity to establish latency in v-kLANA Δ331-495 is markedly less than v-kLANA, but it is higher than v-kLANA Δ332-929. Together, these data indicate that DE internal repeat is required for the establishment of latency in the spleen, and this internal repeat must be present in kLANA in order to have a correct establishment of latency during an in vivo infection.

The infectious center assay results are corroborated by the results of the frequency of viral DNA-positive cells, where the frequencies of v-kLANA and v-kLANA Δ 465-929 were similar (Figure 2C) and v-kLANA Δ332-929 presented a frequency lower than the other viruses. The frequency of viral DNA-positive cells in total splenocytes in v-kLANA Δ331-495 was between frequencies of v-kLANA, v-kLANA Δ 465-929 and v-kLANA Δ332-929.

Despite the deletions, v-kLANA Δ331-495 and v-kLANA Δ465-929 were able to establish latency in the spleen, indicating that they were able to maintain the episome persistence, thus showing that deletions did not affect the kLANA DNA binding domains. It is known that the internal region of kLANA has an important role for episome maintenance (De Leon Vazquez and Kaye, 2011; De Leon Vazquez and Kaye, 2013) and as in v-kLANA Δ331-495 this episome persistence was reduced compared with v-kLANA Δ465-929, this indicated that within the internal region of LANA, the deleted region must exert a more important role in episome maintenance.

All results from v-kLANA Δ332-929 were consistent and are in line with previous work of our laboratory and with in vitro studies made by others (De Leon Vazquez and Kaye, 2013). However, experiments with v-kLANA Δ465-929 need to be repeated.

Future work still needs to be done in order to better understand the functions of the DE internal repeat. First, experiments should be repeated to increase the number of animals to achieve statistical significance. Then, other times after infection may be analysed (for example day 11 and day 21) to assess if the reduced latency at day 14 is in fact just lower magnitude of infection and not altered kinetics of infection.

The molecular functions of the DE repeat region are unknown. However, it is known that many proteins interact with different parts of LANA (De Leon Vazquez and Kaye, 2011), so it would be interesting to perform more protein-protein interaction studies with the aim of identifying more proteins that interact with this specific internal region or within all the internal repeat region. Then, map the localization of those interactions to clarify and unveil the functions of LANA internal repeat region.

This work showed that the DE internal repeat region is required for normal levels of v-kLANA latency. Thus, in the future, the recombinant v-kLANA can be used to test in vitro and then in vivo drugs that target and inhibit the DE region functions in order to try to fight KSHV infection.

References


