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Primary B lymphocytes infected with KSHV can be expanded in vitro and are recognized by LANA-specific CD4+ T cells.

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Abstract

Kaposi sarcoma-associated herpesvirus (KSHV) has a tropism for B lymphocytes in which it establishes latency and can also cause lymphoproliferative disorders of these cells manifesting as primary effusion lymphoma (PEL) and multi-centric Castleman disease (MCD). T cell immunity is vital for the control of KSHV infection and disease however few models of B lymphocyte infection exist to study immune recognition of such cells. Here we developed a model of B lymphocyte infection with KSHV where infected tonsillar B lymphocytes were expanded by providing mitogenic stimuli and these challenged with KSHV-specific CD4+ T cells. Infected cells expressed viral proteins found in PELs namely LANA and vIRF3, albeit at lower levels, with similar patterns of gene expression for the major latency, vIL-6 and vIRF3 transcripts. Despite low level expression of ORF50, transcripts for the immune evasion genes K3 and K5 were detected, with some downregulation of cell surface expressed CD86 and ICAM. The vast majority of infected lymphocytes expressed IgM and the Igλ light chain, recapitulating these features seen in infected cells in MCD. We assessed the ability of the infected lymphocytes to be targeted by a panel of MHC class II matched CD4+ T cells and found that LANA-specific T cells restricted to different epitopes recognized these infected cells. Given that at least some KSHV latent antigens are thought to be poor targets for CD8+ T cells, we suggest that CD4+ T cells are potentially important effectors for the in vivo control of KSHV infected B lymphocytes.

Importance

KSHV establishes a latent reservoir within B lymphocytes but few models exist to study KSHV-infected B cells other than the transformed PEL cell lines which have likely accrued

mutations during the transformation process. We developed a model of KSHV-infected primary B lymphocytes which recapitulate features seen in PEL and MCD by gene expression and cell phenotype analysis, allowing the study of T cell recognition of these cells. Challenge of KSHV-infected B cells with CD4+ T cells specific for LANA, a protein expressed in all KSHV-infected cells and malignancies in vivo, showed that these effectors could efficiently recognize such targets. Given the virus expresses immune evasion genes or use proteins with intrinsic properties such as LANA that minimize epitope recognition by CD8+ T cells, CD4+ T cell immunity to KSHV may be important for maintaining the virus host balance.

Introduction

Kaposi sarcoma-associated herpesvirus (KSHV) is one of the two human γ-herpesviruses with oncogenic potential. This virus has a tropism for endothelial cells where it is associated with the development of Kaposi sarcoma (KS), as well as being tropic for B lymphocytes where it can cause primary effusion lymphoma (PEL) and multi-centric Castleman disease (MCD) (1). The immune response is important for control of KSHV infection as infected patients whose cellular immune response is suppressed either for transplantation or due to untreated HIV infection are at an increased risk of developing KS. Importantly in these scenarios if immune competence is restored through relaxation of immunosuppression or administration of highly active anti-retroviral therapy respectively, regression of KS lesions can be seen, implying an important role for the T cell response in control of the virus and malignancies (2, 3).

KSHV is known to infect CD19+ B lymphocytes (4), but little is known how KSHV-specific T cell control is exercised over infected B lymphocytes. The only model which has been used to examine T cell recognition of infected B lymphocytes comes from studies using PEL derived lines as targets. Here CD8+ T lymphocytes were unable to recognize reporter antigens expressed in PELs thought to be related to their low expression of the transporter associated with antigen processing-1 mRNA, disrupting antigen presentation to these effectors (5). CD4+ T cells specific to the genome maintenance protein LANA, a protein expressed in all infected cells and malignancies, in most cases showed poor if any recognition of PELs (6). This was a consequence of expression of the KSHV gene vIRF3 which, amongst other functions, inhibits expression of the MHC class II transcriptional transactivator CIITA; a protein required for the expression of class II and other genes in this antigen processing pathway (7). Although these studies are performed on cell lines derived from

patients with disease, these lines have likely accrued mutations and may not resemble B cells which the virus maintains latency in. The ability of KSHV-specific T lymphocytes to respond to KSHV infected non-transformed B lymphocytes which are likely to have intact antigen processing pathways is so far largely untested.

Several models of primary B cell infection have been developed to study KSHV infection in these cells. Initial studies used CD40 ligand stimulation of B cells to make them receptive to infection (8), while others have co-cultured B lymphocytes with virus producer cells to allow direct cell to cell virus transfer, or exposed B lymphocytes to concentrated preparations of KSHV (9-12). Such in vitro infected cells express latent transcripts and transiently express selected lytic transcripts and genes, inducing some proliferation of the infected cells but unlike the related γ-herpesvirus Epstein-Barr virus (EBV), does not lead to transformation (10). These models have been informative for studying which cytokines may be induced by infection (13) and examining the potential identity of B lymphocyte targets of KSHV, which are likely IgM+ CD27+ Igλ using cells (10).

However the analysis of immune recognition of ex vivo infected B cells has been limited with studies suggesting that CD4+ T cells may suppress spontaneous lytic replication and encourage latency (11). This mechanism required cell contact, the CD4+ T cells to be activated, but was independent of MHC restriction and so how such restriction might operate in vivo is not clear. Furthermore, how ex vivo infected cells which have stably entered latency may be controlled by KSHV-specific immune effectors has not been tested. To examine these questions we developed a KSHV B cell infection model where we expanded infected cells to ask what viral genes are expressed in infected cells, how infection may modulate immune receptor expression and whether antigen-specific T cells can recognize these targets.

Materials and Methods

Tonsil cell preparations and infections

Tonsil specimens were obtained from patients undergoing routine tonsillectomy to treat chronic tonsillitis. Patients were adolescent or young adults and their tonsils were not inflamed at the time of surgery. All participants gave written informed consent in accordance with the Declaration of Helsinki; ethical approval was granted from the South Birmingham Health Authority Local Research Ethics Committee. Specimens were disaggregated to single cell suspensions by teasing apart the tissue and fine mincing. Mononuclear cells were isolated by purification over a Lymphoprep gradient (Nycomed Pharma), as per the manufacturer's instructions, aliquoted, cryopreserved and stored in liquid nitrogen. DNA was isolated from an aliquot of the tonsillar cells for HLA typing by sequence specific oligonucleotide PCR analysis performed at the Anthony Nolan Trust.

Tonsillar mononuclear cells were infected with KSHV using a protocol similar to one previously described (14). Briefly rKSHV.219 infected Vero cells (VK219; (15)) transduced with a pInducer 20 lentivirus (16) engineered to express ORF50 under the control of the tetracycline promoter, or ORF50 lentivirus transduced Vero cells infected with either the BAC16 derived K5 deletion mutant or its paired revertant virus (17), were seeded in 24 well plates at 50 000 cells per well. After 24 hours, virus replication was induced for 24 hours by the addition of 2 µg/ml doxycycline and 1.25 mM sodium butyrate. Media from these wells was then removed and 500 000 tonsillar mononuclear cells were seeded per well, centrifuged onto the VK219 monolayers and incubated for 48 hours. Parallel mock infections of tonsillar cells were conducted by culturing these either on monolayers of VK219 cells which had been treated with 1 mM phosphonoacetic acid (18) for the previous 30 hours prior to induction and

during the co-culture, or culturing the tonsillar cells in the absence of VK219 cells. B cells were then purified from the mock and KSHV infected cultures using anti-CD19 Dynabeads (LifeTechnologies) and the beads removed using a Detachabead CD19 kit as per the manufacturer's instructions.

Purified B cells were cultured in Iscoves MEM with 100 U/ml IL-4 (Peprotech), penicillin streptomycin, gentamycin and Fungizone (Life Technologies) on monolayers of L cells transduced to express CD40L that had been γ-irradiated (10 000 rads). After 48 hours KSHV-infected cells were selected by addition of puromycin to a final concentration of between 0.1-0.3 μg/ml. B cells were expanded, moved to fresh L cell monolayers weekly and maintained under puromycin selection.

Presence of contaminating VK219 cells in the B lymphocyte cultures was determined by performing semi-quantitative end point PCR assays on 25 ng of DNA extracted from the cultures. The PCR assay detected the neomycin resistance gene encoded by the ORF50 lentivirus used to transduce the VK219 cells. Primers used were neo forward AGG ATC TCC TGT CAT CTC ACC TTG CTC CTG and neo reverse AAG AAC TCG TCA AGA AGG CGA TAG AAG GCG. As controls, transduced VK219 cells were mixed with BJAB cells to give 0.1% and 0.01% VK219 cells and DNA extracted from these mixtures. These controls and DNA from the samples was subject to PCR amplification using different numbers of cycles to detect products.

B cell surface marker expression was assessed by staining cells with antibodies specific to HLA class I, HLA-DR, CD20, CD54, CD86 or appropriate isotype control antibodies (Biolegend). Igλ and Igκ expression was assessed by staining cells with biotinylated antibodies specific to these proteins (Southern Biotech) and these detected by incubating with avidin conjugated APC-Cy7 (Biolegend). Cells were fixed in 2% paraformaldehyde

(eBioscience), analyzed on an LSRII flow cytometer (Becton Dickinson) and data processed using Flowjo (Treestar).

Immunofluorescence staining for LANA protein expression in KSHV infected B cells

Established KSHV infected or mock infected B cell lines were assayed for LANA protein expression by immunofluorescence staining. Cell suspensions were washed in phosphate buffered saline (PBS), dried onto microscope slides and fixed in cold acetone for 10 minutes. Slides were then dried, washed in PBS and stained with either an isotype control rat antibody or a LANA-specific rat antibody (clone LN 53, Advanced Biotechnologies) for 1 hour at 37^{o}C. Slides were washed four times and bound antibody detected using an anti-rat Alexa 568 conjugated antibody (Life Technologies) and incubating for 1 hour at 37^{o}C. Slides were then washed and examined using a Nikon E600 microscope fitted with epifluorescence detection.

Western blot analysis

Cells were lysed in 9M urea with 0.075 M Tris HCl, pH 7.5, sonicated and lysates clarified by centrifugation. Protein concentrations were determined by Bradford assay (Bio-Rad Laboratories) and 20 µg of protein separated by SDS-PAGE and transferred onto nitrocellulose membranes using standard techniques. Blots were probed with antibodies specific for LANA (clone LN53), vIRF3 (CM-A807, Abcam) and actin (AC-74, Sigma-Aldrich). Bound antibodies were detected using the appropriate anti-species peroxidase labelled antibody, followed by detection using an ECL kit (GE Healthcare).

Measurement of viral transcripts

Total RNA was extracted from cells using a NucleoSpin RNA II kit (Macherey Nagel) according to the manufacturer's instructions. An aliquot (1µg) was treated with DNAse I

(Life Technologies) to remove residual genomic DNA, before being reverse-transcribed using Qscript (VWR).

Selected KSHV transcripts were quantified by Taqman qRT-PCR using the primer and probe combinations shown in Table 1. Primer and probe sequences were designed using Primer Express 3.0 (Life Technologies) and were based on the BC-1 KSHV genome sequence (accession number U75698). Primer-probe combinations were selected to avoid known KSHV sequence polymorphisms and assays to detect spliced transcripts were designed to span exon-exon junctions. All Taqman probes (Eurogentec) were modified with FAM and TAMRA at the 5′ and 3′ ends, respectively. Cellular GAPDH mRNA, used as an internal control, was detected using a VIC-labelled commercial assay (Life Technologies, 4310884E).

Amplification reactions were prepared in a final volume of 25 µl containing 1 x Taqman Universal MasterMix II (Life Technologies), 300 nM forward and reverse primers, 200 nM probe, 0.5 µl GAPDH reagent and 5 µl cDNA. PCR amplifications were performed using an ABI 7500 with default thermocycling conditions. All test samples were run in duplicate, while template-negative and RT-negative samples served as controls. To determine the absolute levels of KSHV and GAPDH transcripts, serial dilutions from 1 – 10^5 copies of a plasmid (AQ2) were included in each PCR experiment and used to generate appropriate standard curves. AQ2 was derived from the AQ plasmid (19) by the insertion of a commercially synthesised 1093 bp sequence carrying the contiguous KSHV amplicons (GenScript). All data were analysed using Sequence Detection Software v2.0 (Applied Biosystems) and are reported as copies relative to GAPDH.

Comparisons of gene expression levels between PELs and infected B lymphocytes were performed using the R statistical program (v 3.0.2) (20) on $\log_{10}$ transformed mean values so these were normally distributed as judged using the Shapiro-Wilk test. These transformed

values were then subjected to two sample t-tests to determine differences in transcript levels between PEL lines and infected lymphocytes.

KSHV genome loads

DNA was extracted from cells using a NucleoSpin Tissue kit (Macherey Nagel) and viral genome loads determined by qPCR. KSHV DNA was detected using the vIL-6 primer-probe combination while cellular beta 2 microglobulin (B2m), used as an internal control, was detected using published primers (21). Serial dilutions of AQ2 plasmid and BJAB DNA were used to generate standard curves for vIL6 and B2m, respectively. Data are expressed as KSHV genome copies per cell, assuming two B2m genes per diploid cell.

T cells and recognition experiments

The ability of T cells to recognize KSHV infected targets was performed as described previously, using established T cell clones (6). Briefly, triplicate cultures of 5000 T cells were incubated with 50 000 target cells which were either KSHV-infected or mock infected target B cells, or B cells sensitized with the T cells cognate synthetic peptide-epitope (Mimotopes). Cells were incubated in RPMI-1640 10% FCS for 18 hours and supernatants then harvested from these cultures and assayed for IFN-γ by ELISA (Endogen).

Results

KSHV infection of primary B cells and their propagation.

In a preliminary set of experiments we determined whether we could infect tonsillar derived B cells with rKSHV.219 virus. Unfractionated tonsillar mononuclear cells were infected with KSHV by incubating on monolayers of Vero cells which contained latent rKSHV.219 that had been treated 24 hours previously to induce virus replication. As a mock infection, parallel aliquots of tonsillar cells were incubated on monolayers of induced VK219 cells which had been treated for the previous 30 hours with phosphonoacetic acid to inhibit virus production. After 48 hours co-culture, CD19 expressing B cells were selected, cultured for 72 hours to allow GFP expression from the rKSHV.219 genome, and the proportion of infected cells identified by flow cytometry. Figure 1 shows two representative results of such infections from tonsillectomy patients T46 and T7. Consistent with previous reports (9) we found that these cells could be infected at a low percentage; typically GFP expressing cells would be detected in the range of 0.5-1.6% of B cells.

We next asked whether the infected cells could be expanded by in vitro culture. Previous studies had shown that unlike the related γ-herpesvirus Epstein-Barr virus (EBV), ex vivo infection of B cells with KSHV does not lead to transformation of the B cells (9) but induces some limited proliferation (10). To expand the population of B cells, we delivered a mitogenic stimulus to them by incubating these on CD40-ligand expressing L cells in the presence of IL-4. As rKSHV.219 virus encodes a puromycin resistance gene, we enriched for infected B cells by selection with puromycin. We found that the infected cells were very sensitive to puromycin requiring low doses to enrich these cells which did not obviously affect L cell viability. However compared to mock infected B cells cultured in parallel without puromycin, the infected cells grew slowly, doubling every 4-5 days as compared to

the parallel uninfected B cells which doubled every 72 hours. All subsequent experiments used cells which had undergone at least three to four weeks of selection, which contained between 5% to 50% GFP expressing cells. The presence of contaminating VK219 cells was examined using a semi-quantitative PCR assay to detect the neomycin resistance gene encoded by the ORF50 expressing lentivirus used to transduce these cells. Only DNA extracted from lines derived from two of nine lines, T44b and T48, had detectable sequence at levels less than 0.01% of the population (data not shown). B cell lines could be maintained for a similar length of time compared to mock infected cells, typically for up to 12 weeks, with some lasting >20 weeks. During passaging of these cells we monitored for RFP expression, a gene in rKSHV.219 expressed under the control of the PAN promoter, as a marker of lytic cycle replication and observed few cells, usually less than 5%, expressing this marker in the cultures. To confirm that the B cells were infected with rKSHV.219, we purified these by FACS for GFP expression and conducted immunofluoresecence assays on these cells staining for LANA. Figure 2 shows representative results from one of three sorts, showing the characteristic punctate staining of LANA, reminiscent of the pattern seen in primary effusion lymphomas (22), indicating these KSHV infected cells could be expanded in vitro.

Antigen and gene expression of KSHV infected primary B cells

In the next series of experiments we sought to determine which KSHV genes were expressed in the infected B cells, principally to identify which could be used as immunological targets and also monitor any immune evasion gene expression. We focused on likely latent genes and their products as we observed few RFP expressing cells in our established cultures, suggesting no substantial spontaneous lytic reactivation was occurring. Western blot analysis

was performed on lysates from two tonsillar preparations of rKSHV.219 infected B cells which had been FACS sorted to 90% purity or mock infected B cells maintained on CD40-ligand and IL-4 in parallel. As a control, a lysate from the PEL JSC-1, from which rKSHV.219 was derived was used. Blots were initially probed with antibodies to vCyclin and vFLIP however no expression of these proteins was detected in the JSC-1 or any B cell lysates, nor could we detect expression of the EBV protein EBNA1, found in approximately 80% of PEL lines, in infected B lymphocyte lysates (data not shown). Blots were then sequentially probed for LANA, vIRF3 and actin, the latter as a loading control, and results shown in Figure 3. Infected B cells demonstrated expression of LANA, although at levels lower than seen in JSC-1, while vIRF3 expression in the infected B cells was substantially lower than that in the PELs.

To further characterize these infected B cells we measured viral transcript expression by qRT-PCR using a panel of Taqman based assays we developed. These included the splice variants of transcripts from the major latency locus driven from the constitutive promoter as shown in Figure 4A (23, 24), namely the tricistronic mRNA encoding LANA, vCyclin and vFLIP, the bicistronic mRNA encoding vCyclin and vFLIP and the monocistronic mRNA encoding vFLIP. Specificity of assays which detect spliced transcripts was confirmed by testing against unspliced (genomic) DNA which showed no detection using the bicistronic vCyclin vFLIP assay and weak detection using the monocistronic vFLIP assay, giving a background of 0.13% compared to the unspliced tricistronic message. Assays measuring the PEL expressed vIRF3 and vIL-6 mRNAs were also developed as were assays for three lytic cycle expressed genes including the immediate early expressed lytic switch gene ORF50 (RTA) and two early expressed mRNAs K3 and K5. The location of the amplicons within the genome, primer and probe sequences are shown in Table 1. To validate these assays we tested them on RNA extracted from the PELs BC-1, JSC-1, BCBL-1, VG-1, and BC-3. All

assays were compared to a standard which was a synthetic plasmid containing the PCR amplicons, thereby allowing absolute quantitation of transcript levels within a cell type.

Figure 4B (left panels) shows results of qRT-PCR assays reporting results relative to GAPDH expression, grouping transcripts into either those expressed from the latency locus (LANA, vCyclin, vFLIP), PEL specific transcripts (vIRF3 and vIL-6) and lytic transcripts (ORF50, K3 and K5). Latency locus transcripts showed in most cases that the tricistronic transcript was the most abundant followed by the bicistronic transcript, the exception being BC-1 where this pattern was reversed. Previous northern blot analysis of BC-1 has showed a consistent profile as to what we detected, but some contrast for BCBL-1, where northern analysis indicated the bicistronic transcript to be more abundant than the tricistronic message (23, 25). What dictates splicing of these RNAs is unknown and so why this difference has occurred is not clear, but may relate to drift in gene expression patterns in cultured cells over time or be related to the culture conditions used in our experiments. Interestingly the BCP-1 PEL, not analyzed in our experiments, by northern analysis showed a similar pattern of transcript expression as to what we have detected in most PELs with the tricistronic more abundant than the bicistronic message (26). Low level expression of the monocistronic transcript was detected, consistent with previous findings (24). All PELs expressed vIL-6 RNA, usually at high levels compared to other transcripts, and all expressed vIRF3. In most cases low levels of the ORF50 transcript were detected but perhaps surprisingly, transcripts for the early expressed K3 and K5 genes were detected.

We next performed these qRT-PCR assays on RNA extracted from the infected B cells which had been enriched for infection by selection with puromycin. These cells showed different levels of KSHV infection as judged by GFP expression, thus: T3 90%, T10 90%, T13 75%, T14 43%, T32 51%, T42 62%, T44a 51%, T44b 54% and T48 29%. To account for these variations and allow comparison of transcript levels between the different infected cells and

PELs, we corrected transcript levels to 100% GFP expression. Figure 4B (right panels) shows results of transcript levels in these cells where patterns of transcript expression showed similarities to what was seen in the PELs. Thus the tricistronic was the most abundant of the latency locus transcripts, followed by the bicistronic with very low levels of the monocistronic transcripts detected. The vIL-6 transcripts were again the most abundant detected, while there was lower and variable expression of vIRF3. Low levels of ORF50 transcript were detected, consistent with our observation of few if any RFP expressing cells in the cultures. However similar to the PEL data, some expression of K3 and K5 transcripts was detected in the infected B cells. Statistical analysis comparing individual transcript levels between PELs and infected lymphocytes showed that only the tricistronic transcript showed a significant difference between the two cell types (t-test, $p=0.023$). Overall, the pattern of transcript level in the infected B cells broadly recapitulated what was detected in the PELs.

PEL cell lines are known to harbor high frequencies of KSHV genomes so using our qPCR assays we quantified genome load per cell in the infected B cells and compared these levels to what we observed in PELs. Figure 4C shows results of these assays. PELs contained variable but high levels of genomes with BC-3 containing over 600 copies of the genome, while JSC-1, BCBL-1 and BC-1 had lower yet substantial frequencies of genomes, being between 145 to 244 copies per cell. These values were comparable to those previously reported using a similar methodology on these cell lines (27). Interestingly in repeated assays on VG-1 DNA only two copies of the genome per cell were detected. Analysis of genome loads of infected B cells, corrected for frequency of GFP expressing cells, showed these had lower frequencies as compared to most PELs in five of seven lines analyzed, while lines derived from donors T10 and T44a showed comparable or greater copies of genomes. A second independently established line from this donor, T44b, showed levels closer to the

other infected cells. These findings suggest that that the infected B cells maintain similar or reduced genome loads as compared to PELs.

Cell surface phenotype analysis of infected B cells

We next examined the B cells to identify phenotypic markers associated with infection and whether there was evidence of altered immunological marker expression. Firstly the identity surface immunoglobulin heavy and light chain expression was determined as in MCD, KSHV is found in IgM positive λ light chain expressing cells (28). Here purified B cells which had been exposed to the virus producer cells and then cultured for 72 hours to allow GFP expression were stained for surface expression of Igλ and IgM. Figure 5A shows flow cytometry results of this analysis, where GFP negative uninfected cells showed there was split expression of Igλ and Igκ (not shown) and that the majority of the population expressed IgM. By contrast, few GFP positive cells showed Igκ expression (data not shown), however there was a marked if not exclusive preference for co-expression of Igλ and IgM by the infected cells. Figure 5B shows a similar analysis of established B cell lines which had been maintained for more than 5 months. Mock infected cell lines contained both Igλ and Igκ (not shown) expressing cells, however there were few IgM expressing cells in these cultures. The KSHV infected cells however showed little evidence of Igκ expression (not shown) but expressed Igλ and had maintained their expression of IgM, indicating that culturing these cells in this fashion recapitulates phenotypic features seen in KSHV disease states.

KSHV encodes genes such as K3, K5 and vIRF3 which can modulate cell surface expression of molecules which immune effectors recognize or receive co-stimulatory signals from (7, 29-32). As such we determined the cell surface expression of HLA class I, class II, and the

co-stimulatory molecules CD86 and CD54 (ICAM) on infected cells which had undergone enrichment by puromycin selection and compared expression to co-resident uninfected cells within the culture by flow cytometric analysis. Despite expression of K3 transcripts in infected cells (Figure 4), no obvious modulation of this proteins target, namely surface HLA class I, was observed (Figure 6A). Similarly infected cells showed little change in surface levels of HLA class II as compared to uninfected cells, despite the presence of vIRF3 transcripts.

Analysis of T lymphocyte costimulatory marker expression is presented in Figure 6B where compared to uninfected cells, infected cells had reduced surface levels of CD86 and CD54. Such a profile is consistent with expression of the K5 gene as measured in the qRT-PCR analysis, whose product has ubiquitin ligase activity which induces endocytosis of these proteins. To test whether K5 was responsible for the downregulation of these markers, B cells were infected with a recombinant KSHV in which the K5 gene was deleted (K5Δ) or a derivative of this virus in which the K5 gene is restored (K5R) (17). Cells were selected and surface marker analysis conducted as before; Figure 6C shows CD86 and CD54 surface expression analysis results of one of three pairs of lines infected with the K5Δ or K5R viruses. Cells infected with the K5Δ virus showed substantial but not complete restoration of surface CD86 and CD54 expression as compared to co-resident uninfected cells, suggesting that K5 expression alters at least some surface markers on infected cells in this model.

Recognition of KSHV infected B cells with KSHV-specific CD4+ T cells

As we had demonstrated that the KSHV-infected cells expressed LANA RNA and protein, but did not appear to obviously modulate MHC class II cell surface expression, we determined whether these infected cells could be targeted by a panel of LANA-specific CD4+

T cells. We were especially interested in the ability of CD4+ T cells to recognize this antigen as it is expressed in all infected cells in vivo and the repeat sequences within LANA have been shown to decrease presentation of epitopes to CD8+ T cells (33, 34). For these experiments we HLA-typed the infected B cells and challenged these with HLA matched CD4+ T cell clones specific for LANA which we had previously established (6). A total of six T cell clones were used, three of which were responsive to the HLA-DQ6 presented peptides LAPSTLRSLRKRRLSSPQGP (LAP/LRS), EYRYVLRTSPPHRPG (EYR) or PAFVSSPTLPVAPIP (PAF), two clones which were specific for the HLA-DR13 presented peptides LRSLRKRRLSSPQGP (LRS) or GDDLHLQPRRKHVAD (GDD), and one responsive to the HLA-DQ7 presented peptide GSPTVFTSGLPAFVS (GSP). Here the clones were incubated with either the infected B cells, mock infected B cells or mock infected B cells sensitized with the T cells cognate peptide, for 18 hours and recognition assessed by measuring IFN-γ secretion from the T cells. Figure 7 shows representative results from three assays, where the effectors were challenged with B cell lines infected with KSHV or mock infected lines that had been maintained on CD40L and IL-4 in parallel. T7 and T31 KSHV-infected targets were 68% and 93% GFP positive respectively and these co-expressed both HLA-DR13 and HLA-DQ6, while T10 infected targets were 23% GFP positive and expressed HLA-DQ7. In all cases we observed IFN-γ secretion from the T cells when challenged with the infected cells, indicating that despite the down regulation of cell surface co-stimulatory molecules and the low level expression of vIRF3, these KSHV LANA-specific CD4+ T cells could recognize the KSHV-infected B cells.

Discussion

In the present study we developed a model of primary B lymphocyte infection with KSHV to examine gene expression and immune recognition of infected B cells which, unlike PEL cell lines, have not gone through a transformation process. Infected B lymphocytes expanded with mitogenic stimuli expressed latent proteins and genes in a similar pattern to what is observed in PELs. Phenotypically these recapitulated features of MCD infected cells namely the almost exclusive expression of IgM and Igλ by the infected cells. Although these cells expressed genes associated with immune evasion, some of which did modulate cell surface markers, we found that the KSHV-infected cells were capable of being recognized by LANA-specific CD4+ T cells.

Our initial studies characterizing the KSHV-infected B cells showed that they expressed key proteins namely LANA and vIRF3, while qRT-PCR analysis indicated that a similar repertoire of genes were expressed as seen in PELs assayed in parallel. However despite finding transcripts for LANA and vIRF3 in the infected lymphocytes, the corresponding levels of protein for each were lower than the PELs, suggesting that in this model, factors other than transcript abundance affects protein levels. We have previously found that ectopic expression of LANA or vIRF3 in model cell lines at levels similar to those in PELs is toxic (unpublished observations). We speculate that PELs adapt to high level expression of these proteins during the transformation process and that the infected lymphocytes in the present study have not had sufficient time to adapt to high level protein expression. Some expression of the lytic cycle assigned K3 and K5 genes was also observed, potentially related to the low level expression of ORF50. However few cells expressed RFP, a marker of lytic cycle replication, suggesting that either these transcripts were detected from the few cells with lytic virus replication, or that these genes were being expressed outside of true lytic replication.

428 The latter case seems most likely as all infected lymphocytes, rather than a subset, showed
429 decreased surface expression of K5 target proteins. Furthermore, recent transcriptome
430 analysis of the PEL BCP-1 indicated that some transcripts, particularly K5, can be found in
431 these cells in the absence of obvious lytic cycle replication (35). Such expression patterns
432 show some contrast to what has been observed in latently infected SLK cells, where there is
433 minimal expression of K5 and modulation of its targets outside of lytic cycle replication (17).
434 K5 expression may then have been induced in our cells due to the culture conditions used, or
435 as PELs express this transcript, the K5 promoter may be activated in a B cell background, due
436 to B cell specific transcription factors. Alternatively this may be a consequence of the
437 elevated genome loads detected as high genome loads, at least in primary infection of cells,
438 are associated with the modulation of these cell surface immune cell stimulating ligands (36).

439 Despite the expression of K5 and the decreased surface expression of CD86 and CD54, no
440 obvious modulation of surface MHC class I was seen on the infected cells. This is likely due
441 to the apparent increased sensitivity of CD86 and CD54 to K5 as compared to MHC class I,
442 especially certain allotypes of class I (29, 31, 37). Turning to MHC class II expression, no
443 obvious decrease in cell surface expression of these proteins was observed on infected cells
444 despite the expression of vIRF3. This protein can block promoter activity of the class II
445 transcriptional transactivator CIITA, which is required for expression of MHC class II and
446 other genes required for the MHC class II processing pathway (7). Although vIRF3
447 transcript was expressed, we detected low levels of protein in infected cells compared to the
448 JSC-1 PEL. Interesting in this context we have previously found that BCBL-1 PELs
449 similarly express low levels of vIRF3 protein compared to other PELs, but maintain good
450 expression of surface MHC class II (6).

451 Analysis of cell surface marker expression showed that the vast majority of KSHV infected
452 cells were predominantly within the Igλ light chain IgM heavy chain subset, reminiscent of

these features observed within infected cells of MCD lesions (28). Established cultures of infected cells maintained this phenotype while parallel mock infected cultures switched immunoglobulin heavy chain isotype usage, a likely consequence of continual CD40-ligand and IL-4 stimulation, suggesting that virus infection suppresses isotype switching. Interestingly this bias of light chain usage was seen within 72 hours after virus infection. Related studies in which KSHV infection of tonsillar B lymphocytes was examined at 72 hours post exposure for LANA protein expression have also shown that the vast majority of LANA expressing cells were in the Igλ using population (10). Why this bias occurs is unclear as there are no obvious functional or phenotypic differences between these two subsets. Potentially this selection may be a consequence of vFLIP expression as B cells within a transgenic vFLIP mouse model more frequently utilize this light chain (38). In this case the ability of vFLIP to promote expression of NF-kB may be responsible, as this transcription factor is required at key stages of immature B cell development for the selection of Igλ using cells (reviewed in (39)). However as light chain rearrangement occurs during B lymphocyte maturation in the bone marrow, the tonsillar B lymphocytes used in this study likely have already undergone light chain selection. This may suggest that either infected cells may be converted to Igλ usage through processes such as receptor revision (40) although this may seem unlikely given the vast majority of cells are expressing this receptor by 72 hours post infection. Alternatively Igκ using cells may not sustain infection.

The T lymphocyte response is vital for effective control of KSHV infection and disease, however whether one subset of T lymphocytes, either CD4+ or CD8+, is more effective remains an open question. This is particularly relevant when examining features of proteins such as the genome maintenance protein LANA, which is expressed in all KSHV-infected cells and malignancies, making it an attractive immunological target. Like its related genome maintenance protein homologue in EBV, EBNA1, LANA encodes extensive repeat

sequences, some of which share nucleotide homology with EBNA1. These function to limit protein synthesis *in cis* and inhibit proteasomal degradation (34, 41-43). As most CD8+ T cell epitopes appear to be derived from newly synthesized proteins which are degraded by the proteasome (44), these features of LANA have been shown to minimize CD8+ T cell recognition of model epitopes inserted into LANA (33, 34, 41). Although LANA-specific CD8+ T cell recognition of KSHV-infected cells is untested, one may predict recognition to be minimal. By contrast, epitope generation for CD4+ T cell recognition would not be subject to these restrictions and as B lymphocytes express class II MHC, these may be legitimate targets for LANA-specific CD4+ T cells. Indeed we found that for all T cell specificities tested, infected cells could be recognized by the MHC matched T cell clones. The magnitude of responses are comparable to those seen in analogous experiments using EBV-specific CD4+ T cells targeting epitopes from some latent antigens such as the EBNA3 proteins expressed by EBV transformed B lymphoblastoid cell lines (LCLs) (45-48).

The ability of the LANA-specific CD4+ T cell clones to recognize KSHV-infected cells shows some contrast to what is seen using EBV EBNA1-specific CD4+ T cells challenged with their cognate LCL which naturally expresses EBNA1. These effectors either do not or weakly recognize such targets (47, 49). This is thought to be a consequence of the poor release of EBNA1 protein by LCLs, thereby restricting the ability of cells to take up antigen and re-present it to T cells, or making EBNA1 epitope presentation reliant on endogenous processing of antigen through a macroautophagy route (48, 50, 51). We have previously shown that B cells with intact antigen processing pathways exogenously fed LANA protein can take up, process and present epitopes to LANA-specific CD4+ T cells and that such B cells can also present endogenously expressed LANA (6). These findings suggest that LANA, despite performing a similar function and having some sequence homology to EBNA1 is processed and presented differently and may be a more relevant target for CD4 T

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503 cells as compared to EBNA1 in EBV infection. Furthermore, we suggest that as LANA has
504 properties which limit efficient CD8+ T cell recognition, CD4+ T cell immunity to this
505 protein is likely to be important in control of KSHV infection and disease.

506

References

1. **Bouvard V, Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F, Benbrahim-Tallaa L, Guha N, Freeman C, Galichet L, Cogliano V.** 2009. A review of human carcinogens--Part B: biological agents. Lancet Oncol **10:**321-322.
2. **Bihl F, Mosam A, Henry LN, Chisholm JV, 3rd, Dollard S, Gumbi P, Cassol E, Page T, Mueller N, Kiepiela P, Martin JN, Coovadia HM, Scadden DT, Brander C.** 2007. Kaposi's sarcoma-associated herpesvirus-specific immune reconstitution and antiviral effect of combined HAART/chemotherapy in HIV clade C-infected individuals with Kaposi's sarcoma. AIDS **21:**1245-1252.
3. **Duman S, Toz H, Asci G, Alper S, Ozkahya M, Unal I, Celik A, Ok E, Basci A.** 2002. Successful treatment of post-transplant Kaposi's sarcoma by reduction of immunosuppression. Nephrol Dial Transplant **17:**892-896.
4. **Ambroziak JA, Blackbourn DJ, Herndier BG, Glogau RG, Gullett JH, McDonald AR, Lennette ET, Levy JA.** 1995. Herpes-like sequences in HIV-infected and uninfected Kaposi's sarcoma patients. Science **268:**582-583.
5. **Brander C, Suscovich T, Lee Y, Nguyen PT, O'Connor P, Seebach J, Jones NG, van Gorder M, Walker BD, Scadden DT.** 2000. Impaired CTL recognition of cells latently infected with Kaposi's sarcoma-associated herpes virus. J Immunol **165:**2077-2083.
6. **Sabbah S, Jagne YJ, Zuo J, de Silva T, Ahasan MM, Brander C, Rowland-Jones S, Flanagan KL, Hislop AD.** 2012. T-cell immunity to Kaposi sarcoma-associated herpesvirus: recognition of primary effusion lymphoma by LANA-specific CD4+ T cells. Blood **119:**2083-2092.
7. **Schmidt K, Wies E, Neipel F.** 2011. Kaposi's sarcoma-associated herpesvirus viral interferon regulatory factor 3 inhibits gamma interferon and major histocompatibility complex class II expression. J Virol **85:**4530-4537.
8. **Rappocciolo G, Hensler HR, Jais M, Reinhart TA, Pegu A, Jenkins FJ, Rinaldo CR.** 2008. Human herpesvirus 8 infects and replicates in primary cultures of activated B lymphocytes through DC-SIGN. J Virol **82:**4793-4806.
9. **Myoung J, Ganem D.** 2011. Infection of primary human tonsillar lymphoid cells by KSHV reveals frequent but abortive infection of T cells. Virology **413:**1-11.
10. **Hassman LM, Ellison TJ, Kedes DH.** 2011. KSHV infects a subset of human tonsillar B cells, driving proliferation and plasmablast differentiation. J Clin Invest **121:**752-768.
11. **Myoung J, Ganem D.** 2011. Active lytic infection of human primary tonsillar B cells by KSHV and its noncytolytic control by activated CD4+ T cells. J Clin Invest **121:**1130-1140.
12. **Blackbourn DJ, Lennette E, Klencke B, Moses A, Chandran B, Weinstein M, Glogau RG, Witte MH, Way DL, Kutzkey T, Herndier B, Levy JA.** 2000. The restricted cellular host range of human herpesvirus 8. AIDS **14:**1123-1133.
13. **Knowlton ER, Rappocciolo G, Piazza P, Lepone LM, Nadgir SV, Bullotta A, Berendam SJ, Li J, Reinhart TA, Jenkins FJ, Rinaldo CR.** 2014. Human herpesvirus 8 induces polyfunctional B lymphocytes that drive Kaposi's sarcoma. MBio **5:**e01277-01214.
14. **Myoung J, Ganem D.** 2011. Infection of lymphoblastoid cell lines by Kaposi's sarcoma-associated herpesvirus: critical role of cell-associated virus. J Virol **85:**9767-9777.
15. **Vieira J, O'Hearn PM.** 2004. Use of the red fluorescent protein as a marker of Kaposi's sarcoma-associated herpesvirus lytic gene expression. Virology **325:**225-240.
16. **Meerbrey KL, Hu G, Kessler JD, Roarty K, Li MZ, Fang JE, Herschkowitz JI, Burrows AE, Ciccia A, Sun T, Schmitt EM, Bernardi RJ, Fu X, Bland CS, Cooper TA, Schiff R, Rosen JM, Westbrook TF, Elledge SJ.** 2011. The pINDUCER lentiviral toolkit for inducible RNA interference in vitro and in vivo. Proc Natl Acad Sci U S A **108:**3665-3670.
17. **Brulois KF, Chang H, Lee AS, Ensser A, Wong LY, Toth Z, Lee SH, Lee HR, Myoung J, Ganem D, Oh TK, Kim JF, Gao SJ, Jung JU.** 2012. Construction and manipulation of a new Kaposi's sarcoma-associated herpesvirus bacterial artificial chromosome clone. J Virol **86:**9708-9720.

18. **Sun R, Lin SF, Staskus K, Gradoville L, Grogan E, Haase A, Miller G.** 1999. Kinetics of Kaposi's sarcoma-associated herpesvirus gene expression. J Virol **73:**2232-2242.
19. **Tierney RJ, Shannon-Lowe CD, Fitzsimmons L, Bell AI, Rowe M.** 2015. Unexpected patterns of Epstein-Barr virus transcription revealed by a high throughput PCR array for absolute quantification of viral mRNA. Virology **474:**117-130.
20. **R Development Core Team.** 2015. R: A language and environment for statistical computing., R Foundation for Statistical Computing, http://www.R-project.org.
21. **Junying J, Herrmann K, Davies G, Lissauer D, Bell A, Timms J, Reynolds GM, Hubscher SG, Young LS, Niedobitek G, Murray PG.** 2003. Absence of Epstein-Barr virus DNA in the tumor cells of European hepatocellular carcinoma. Virology **306:**236-243.
22. **Gao SJ, Kingsley L, Li M, Zheng W, Parravicini C, Ziegler J, Newton R, Rinaldo CR, Saah A, Phair J, Detels R, Chang Y, Moore PS.** 1996. KSHV antibodies among Americans, Italians and Ugandans with and without Kaposi's sarcoma. Nat Med **2:**925-928.
23. **Sarid R, Wiezorek JS, Moore PS, Chang Y.** 1999. Characterization and cell cycle regulation of the major Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) latent genes and their promoter. J Virol **73:**1438-1446.
24. **Grundhoff A, Ganem D.** 2001. Mechanisms governing expression of the v-FLIP gene of Kaposi's sarcoma-associated herpesvirus. J Virol **75:**1857-1863.
25. **Dittmer D, Lagunoff M, Renne R, Staskus K, Haase A, Ganem D.** 1998. A cluster of latently expressed genes in Kaposi's sarcoma-associated herpesvirus. J Virol **72:**8309-8315.
26. **Talbot SJ, Weiss RA, Kellam P, Boshoff C.** 1999. Transcriptional analysis of human herpesvirus-8 open reading frames 71, 72, 73, K14, and 74 in a primary effusion lymphoma cell line. Virology **257:**84-94.
27. **Han SJ, Marshall V, Barsov E, Quinones O, Ray A, Labo N, Trivett M, Ott D, Renne R, Whitby D.** 2013. Kaposi's sarcoma-associated herpesvirus microRNA single-nucleotide polymorphisms identified in clinical samples can affect microRNA processing, level of expression, and silencing activity. J Virol **87:**12237-12248.
28. **Dupin N, Diss TL, Kellam P, Tulliez M, Du MQ, Sicard D, Weiss RA, Isaacson PG, Boshoff C.** 2000. HHV-8 is associated with a plasmablastic variant of Castleman disease that is linked to HHV-8-positive plasmablastic lymphoma. Blood **95:**1406-1412.
29. **Coscoy L, Ganem D.** 2001. A viral protein that selectively downregulates ICAM-1 and B7-2 and modulates T cell costimulation. J Clin Invest **107:**1599-1606.
30. **Ishido S, Wang C, Lee BS, Cohen GB, Jung JU.** 2000. Downregulation of major histocompatibility complex class I molecules by Kaposi's sarcoma-associated herpesvirus K3 and K5 proteins. J Virol **74:**5300-5309.
31. **Ishido S, Choi JK, Lee BS, Wang C, DeMaria M, Johnson RP, Cohen GB, Jung JU.** 2000. Inhibition of natural killer cell-mediated cytotoxicity by Kaposi's sarcoma-associated herpesvirus K5 protein. Immunity **13:**365-374.
32. **Timms RT, Duncan LM, Tchasovnikarova IA, Antrobus R, Smith DL, Dougan G, Weekes MP, Lehner PJ.** 2013. Haploid genetic screens identify an essential role for PLP2 in the downregulation of novel plasma membrane targets by viral E3 ubiquitin ligases. PLoS Pathog **9:**e1003772.
33. **Zaldumbide A, Ossevoort M, Wiertz EJ, Hoeben RC.** 2007. In cis inhibition of antigen processing by the latency-associated nuclear antigen I of Kaposi sarcoma herpes virus. Mol Immunol **44:**1352-1360.
34. **Kwun HJ, da Silva SR, Qin H, Ferris RL, Tan R, Chang Y, Moore PS.** 2011. The central repeat domain 1 of Kaposi's sarcoma-associated herpesvirus (KSHV) latency associated-nuclear antigen 1 (LANA1) prevents cis MHC class I peptide presentation. Virology **412:**357-365.
35. **Cao S, Strong MJ, Wang X, Moss WN, Concha M, Lin Z, O'Grady T, Baddoo M, Fewell C, Renne R, Flemington EK.** 2015. High-throughput RNA sequencing-based virome analysis of 50 lymphoma cell lines from the Cancer Cell Line Encyclopedia project. J Virol **89:**713-729.

36. **Adang LA, Tomescu C, Law WK, Kedes DH.** 2007. Intracellular Kaposi's sarcoma-associated herpesvirus load determines early loss of immune synapse components. J Virol **81:**5079-5090.
37. **Thomas M, Boname JM, Field S, Nejentsev S, Salio M, Cerundolo V, Wills M, Lehner PJ.** 2008. Down-regulation of NKG2D and NKp80 ligands by Kaposi's sarcoma-associated herpesvirus K5 protects against NK cell cytotoxicity. Proc Natl Acad Sci U S A **105:**1656-1661.
38. **Ballon G, Chen K, Perez R, Tam W, Cesarman E.** 2011. Kaposi sarcoma herpesvirus (KSHV) vFLIP oncoprotein induces B cell transdifferentiation and tumorigenesis in mice. J Clin Invest **121:**1141-1153.
39. **Kaileh M, Sen R.** 2012. NF-kappaB function in B lymphocytes. Immunol Rev **246:**254-271.
40. **Seagal J, Melamed D.** 2002. Role of receptor revision in forming a B cell repertoire. Clin Immunol **105:**1-8.
41. **Kwun HJ, da Silva SR, Shah IM, Blake N, Moore PS, Chang Y.** 2007. Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen 1 mimics Epstein-Barr virus EBNA1 immune evasion through central repeat domain effects on protein processing. J Virol **81:**8225-8235.
42. **Yin Y, Manoury B, Fahraeus R.** 2003. Self-inhibition of synthesis and antigen presentation by Epstein-Barr virus-encoded EBNA1. Science **301:**1371-1374.
43. **Levitskaya J, Sharipo A, Leonchiks A, Ciechanover A, Masucci MG.** 1997. Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen 1. Proc Natl Acad Sci U S A **94:**12616-12621.
44. **Yewdell JW.** 2011. DRiPs solidify: progress in understanding endogenous MHC class I antigen processing. Trends Immunol **32:**548-558.
45. **Long HM, Haigh TA, Gudgeon NH, Leen AM, Tsang CW, Brooks J, Landais E, Houssaint E, Lee SP, Rickinson AB, Taylor GS.** 2005. CD4+ T-cell responses to Epstein-Barr virus (EBV) latent-cycle antigens and the recognition of EBV-transformed lymphoblastoid cell lines. J Virol **79:**4896-4907.
46. **Long HM, Zuo J, Leese AM, Gudgeon NH, Jia H, Taylor GS, Rickinson AB.** 2009. CD4+ T-cell clones recognizing human lymphoma-associated antigens: generation by in vitro stimulation with autologous Epstein-Barr virus-transformed B cells. Blood **114:**807-815.
47. **Taylor GS, Long HM, Haigh TA, Larsen M, Brooks J, Rickinson AB.** 2006. A role for intercellular antigen transfer in the recognition of EBV-transformed B cell lines by EBV nuclear antigen-specific CD4+ T cells. J Immunol **177:**3746-3756.
48. **Leung CS, Haigh TA, Mackay LK, Rickinson AB, Taylor GS.** 2010. Nuclear location of an endogenously expressed antigen, EBNA1, restricts access to macroautophagy and the range of CD4 epitope display. Proc Natl Acad Sci U S A **107:**2165-2170.
49. **Mautner J, Pich D, Nimmerjahn F, Milosevic S, Adhikary D, Christoph H, Witter K, Bornkamm GW, Hammerschmidt W, Behrends U.** 2004. Epstein-Barr virus nuclear antigen 1 evades direct immune recognition by CD4+ T helper cells. Eur J Immunol **34:**2500-2509.
50. **Long HM, Chagoury OL, Leese AM, Ryan GB, James E, Morton LT, Abbott RJ, Sabbah S, Kwok W, Rickinson AB.** 2013. MHC II tetramers visualize human CD4+ T cell responses to Epstein-Barr virus infection and demonstrate atypical kinetics of the nuclear antigen EBNA1 response. J Exp Med **210:**933-949.
51. **Paludan C, Schmid D, Landthaler M, Vockerodt M, Kube D, Tuschl T, Munz C.** 2005. Endogenous MHC class II processing of a viral nuclear antigen after autophagy. Science **307:**593-596.

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660

661 Figure legends

662 Figure 1. Frequency of KSHV-infected B cells after ex vivo infection. Tonsillar B cells from
663 donors T46 and T7 were either infected with KSHV by culturing on monolayers of induced
664 VK219 cells for 48 hours, or mock infected by culturing on induced monolayers which had
665 been pre-treated with phosphonoacetic acid (PAA) to inhibit virus replication. B
666 lymphocytes were then magnetically sorted, cultured for a further 72 hours to allow
667 expression of the GFP reporter from the rKSHV.219 genome, then stained for CD20 surface
668 expression and the proportion of cells expressing GFP as a marker of infection determined by
669 flow cytometry.

670 Figure 2. Expression of LANA protein in B cell lines by immunofluorescent staining
671 analysis. Mock infected or KSHV-infected cells which had been established for eight weeks
672 were stained with a LANA-specific monoclonal antibody and bound antibody detected by
673 staining with an Alexa-fluor conjugated secondary antibody. Antibody binding was
674 visualized by epifluorescence microscopy.

675 Figure 3. Western blot analysis for detection of viral proteins in lysates from B cell lines.
676 Proteins from lysates of the PEL JSC-1, mock infected and KSHV-infected B cell lines which
677 had been maintained for 18 weeks were separated by PAGE and blotted onto PVDF

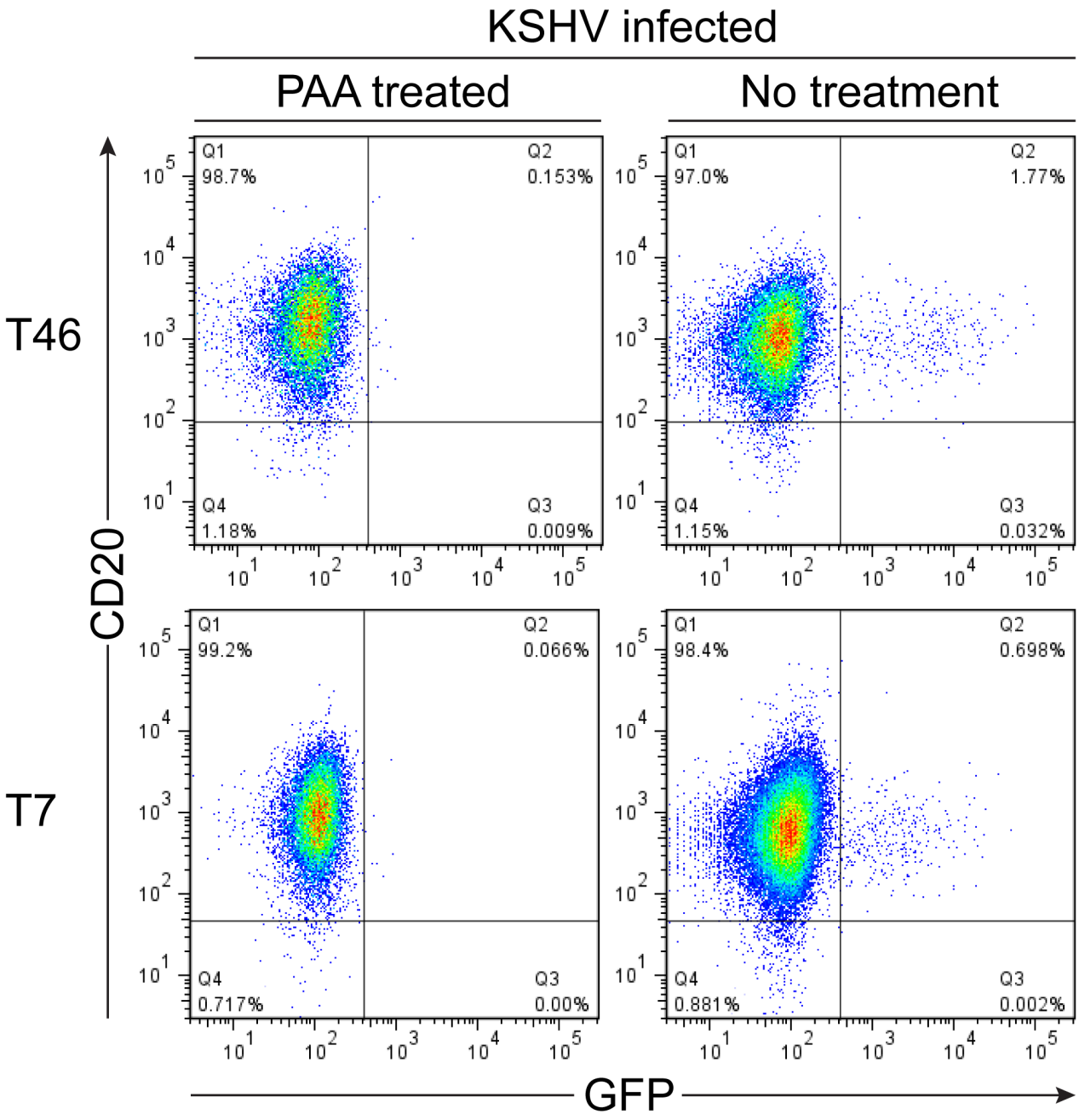
membranes. Blots were sequentially probed for either LANA, vIRF3 or actin. Filled arrowheads represent specific protein detection.

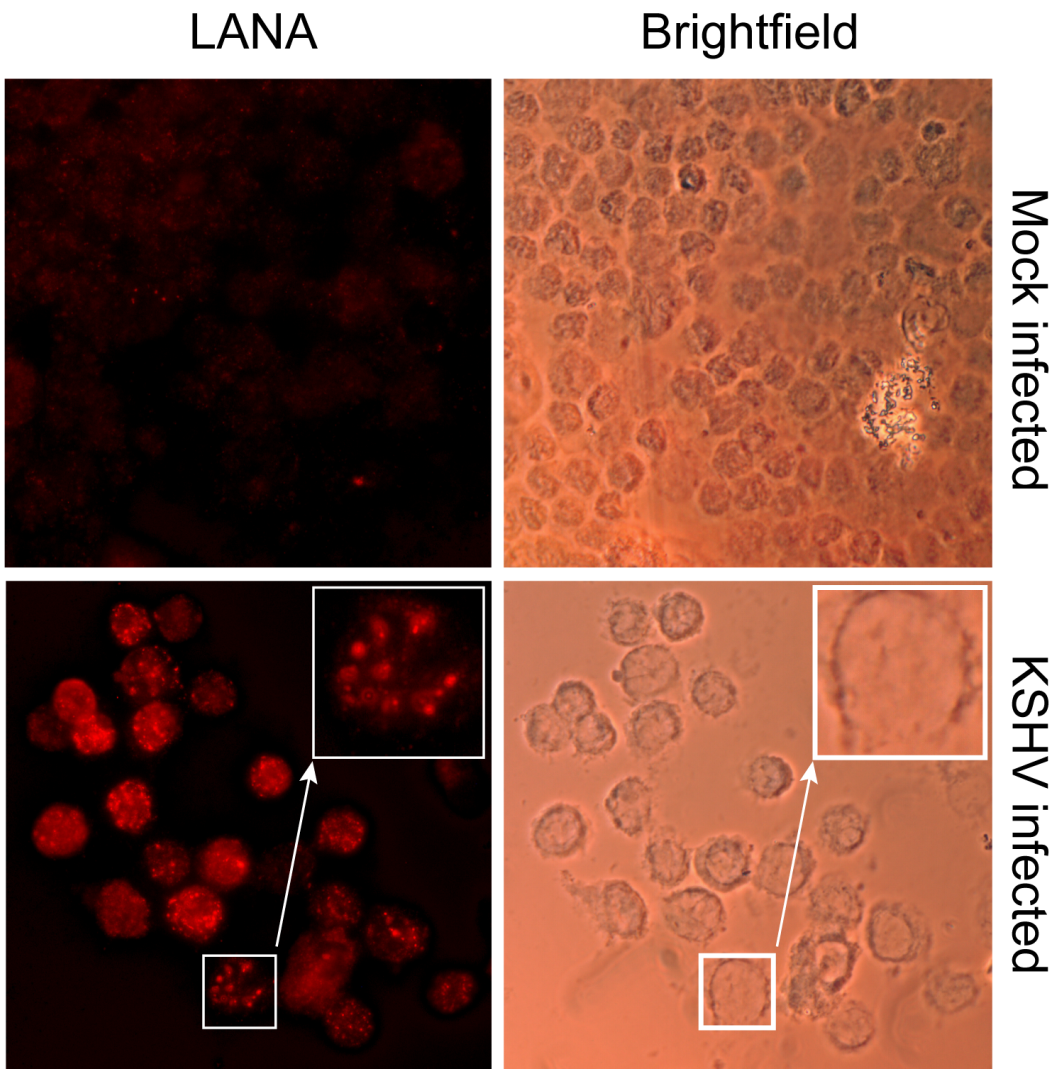
Figure 4. Gene expression analysis and genome load within KSHV infected cell lines. A. Map of qPCR assays to detect splice variants driven from the constitutively active promoter of the latency locus. Arrows represent primers and dashed lines indicate intronic regions spanned by the primers. B. Gene expression was determined from cDNA prepared from the PEL lines BC-1, JSC-1, BCBL-1, VG-1 and BC-3 and the infected B cell lines, assayed for expression of the following transcripts: tricistronic LANA-vCyclin-vFLIP, bicistronic vCyclin-vFLIP, monocistronic vFLIP, vIL-6, vIRF3, K3, K5, ORF50 and GAPDH. Transcripts are expressed relative to GAPDH transcript abundance. * denotes $<10^{-5}$ vFLIP transcript detected for T3 and T10. C. Genome load was determined in DNA extracted from the above cell lines and quantified using the vIL-6 qPCR assay. Results shown for the infected B cell lines have been corrected to represent 100% GFP expression. Infected lymphocytes had been established for the following times: T3 18 weeks, T10 18 weeks, T13 10 weeks, T14 8 weeks, T32 10 weeks, T42 12 weeks, T44a 10 weeks, T44b 8 weeks, T48 6 weeks.

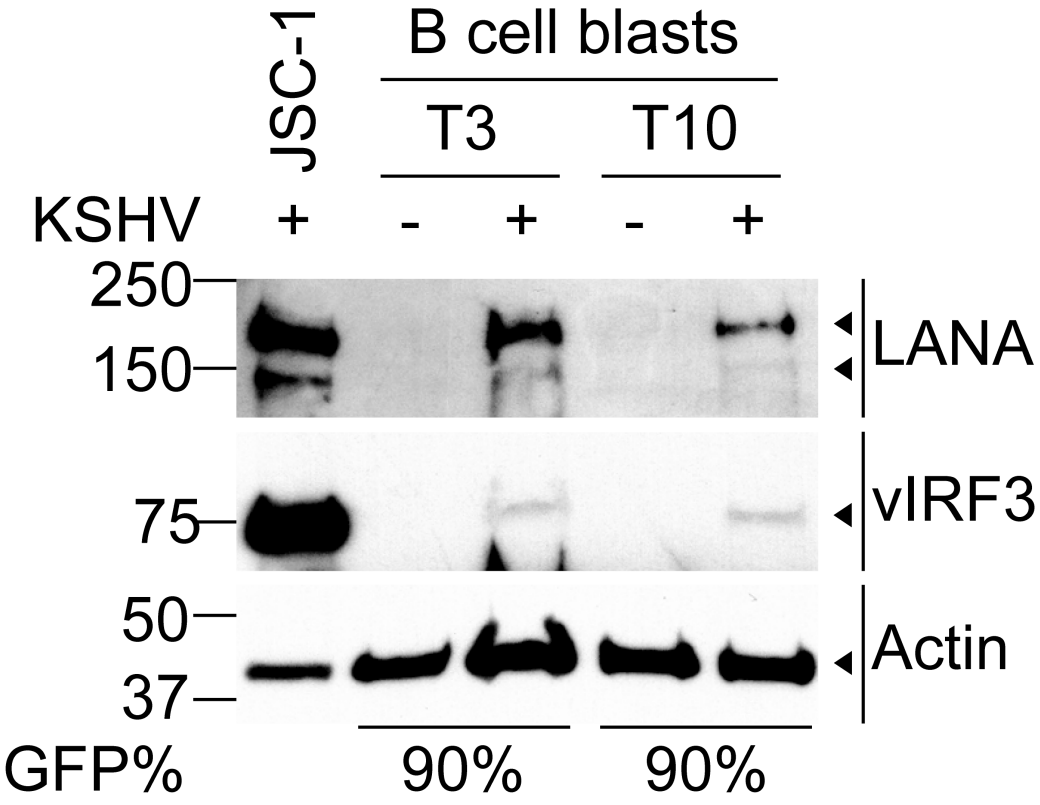
Figure 5. Cell surface immunoglobulin usage by KSHV-infected B lymphocytes. A. Tonsillar cells were infected with KSHV as described in Figure 1 and stained for the expression of CD20, Igλ and IgM, followed by flow cytometry analysis. Populations shown are gated on the CD20 GFP negative uninfected cells or CD20 GFP positive infected population. B. B cell lines which were either mock- or KSHV-infected and maintained for twenty weeks were analyzed for cell surface expression of Igλ and IgM. Mock infected cells are gated on viable cells, while KSHV infected cells are gated on GFP viable cells.

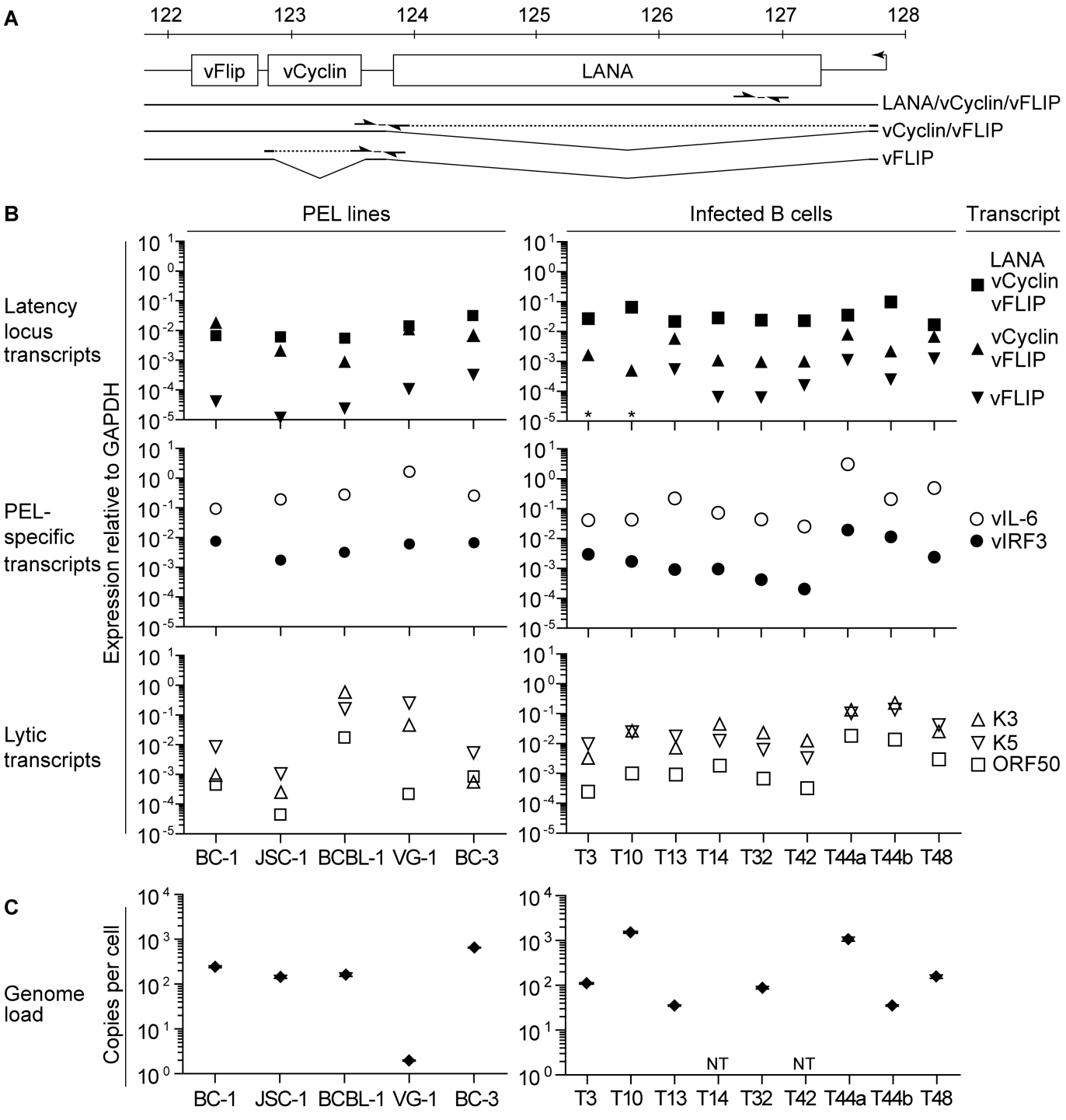
Figure 6. Flow cytometric analysis of cell surface expressed immunological markers on KSHV-infected B cell lines. A. KSHV-infected B cell lines which had been established for eight weeks were stained with antibodies specific to either MHC class I or class II or an isotype control antibody (Iso) and gated on either GFP positive infected cells (KSHV) or GFP negative cells co-resident in the culture (Non). B. KSHV-infected B cell lines were stained for expression of CD86 or CD54 and gated on infected and co-resident non-infected cells as before. C. KSHV-infected cell lines that had been established for six weeks were infected with either a K5 deleted virus (K5Δ) or a virus derived from this construct in which the K5 gene was restored (K5R) and stained for expression of CD86 or CD54 on infected and co-resident non-infected cells as before. Data is representative of at least three independent cell lines for each assay.

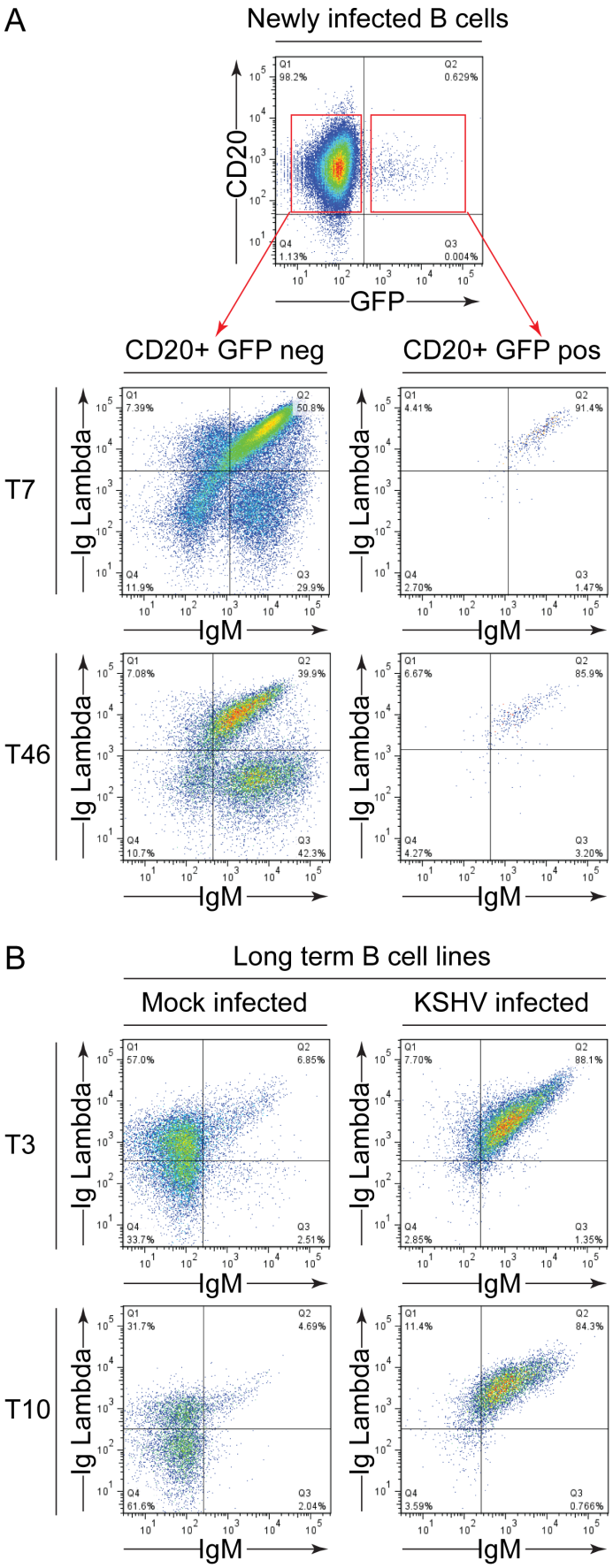
Figure 7. LANA-specific CD4+ T cell recognition of mock- or KSHV-infected B lymphocyte lines. A. KSHV-infected B cell lines or mock infected B cell lines or mock infected B cell lines sensitized with the T cells cognate peptide (Pep) derived from donors T7 and T31 which co-expressed HLA-DQ6 and HLA-DR13 were challenged with the LANA-specific HLA-DQ6 restricted clones LAP/LRS, EYR, PAF or the DR13 restricted clones LRS and GDD. B lymphocyte lines had been established for ten weeks. B. KSHV-infected or mock infected B cell lines derived from donor T10 which expressed HLA-DQ7 were challenged with the LANA-specific HLA-DQ7 restricted clone GSP. B lymphocyte lines had been established for eight weeks. In both cases T cell recognition of targets was assessed by measuring IFN-γ secretion. Error bars represent the standard deviation of assay replicates.

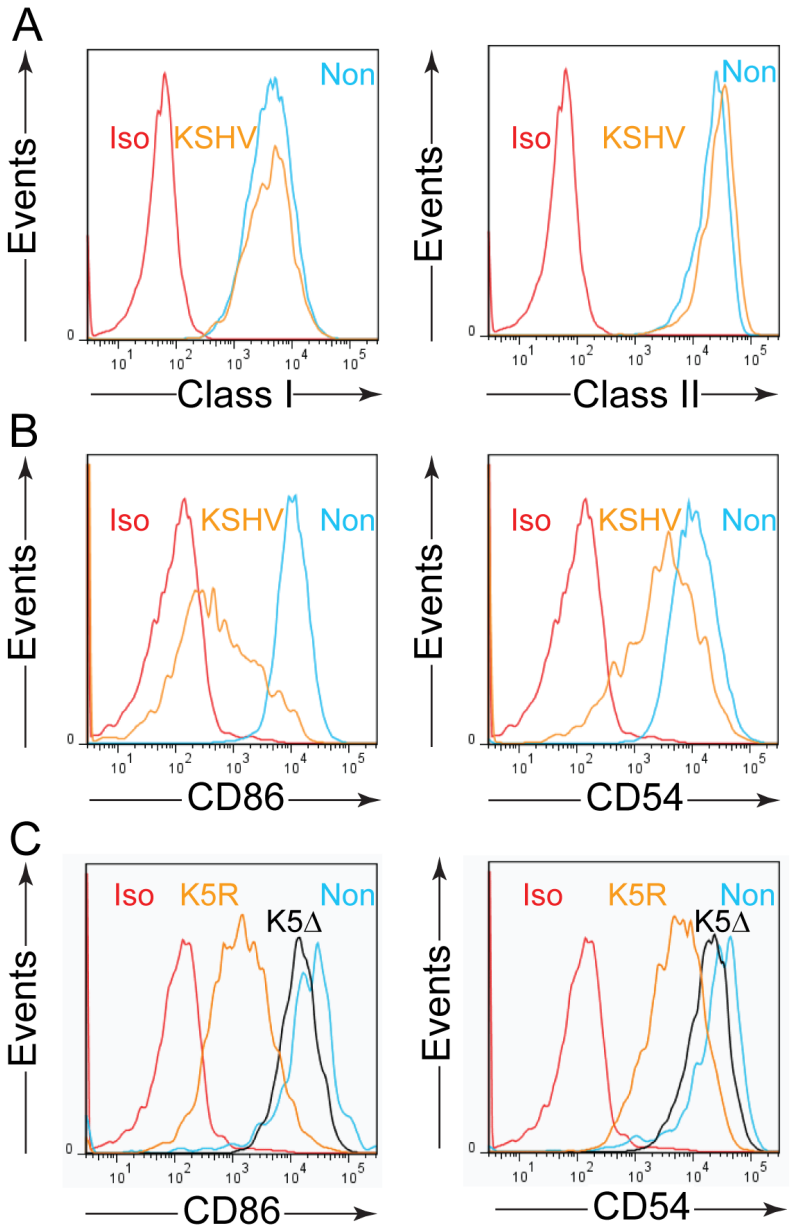



LANA
Brightfield
Mock infected
KSHV infected









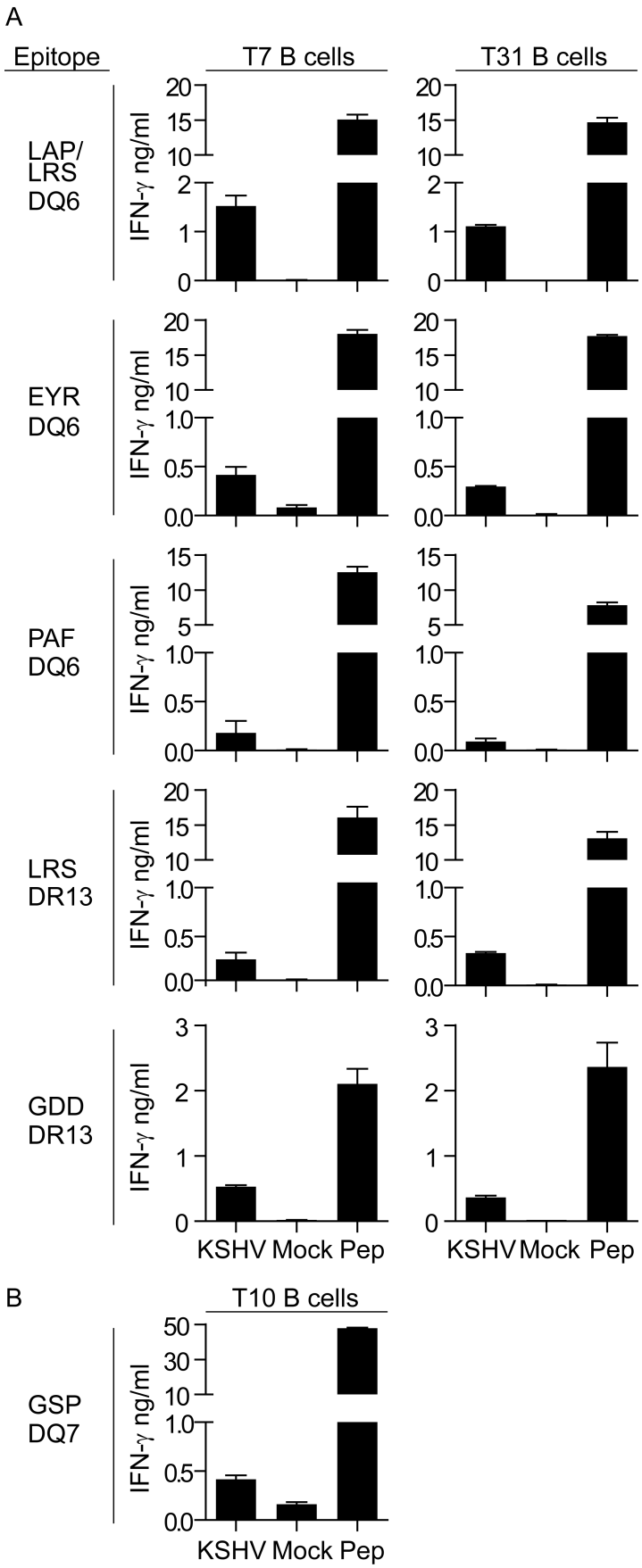


Table 1 qPCR assay primer and probe sequences with BC-1 genomic locations

Assay	Forward primer	Probe	Reverse primer
LANA/	TTTACCTCCACCGGCACTCT	CACGTCTTCCTCCCCAATCCCTCC	GTCCCCGGAGACACAGGAT
vCyclin/	127007-126988	126974-126951	126927-126945
vFLIP			
vCyclin/	GGTAGATGGGTCGTGAGAACACT	ACCGTCGCCGCTCCGCACTT	TCCGGCTGACTTATAAACA^AAGC
vFLIP	123692-123714	123771-123752	127831-127813^123776-123773
vFLIP	TTCCACTGCCGC^CTGTAGAG	TGTCAGGTTCTCCCATCGACGACG	ACGGACAACGGCTAGCGTACT
	122848-122859^123595-123602	123623-123646	123679-123659
vIL-6	GATGCTATGGGTGATCGATGAA	TTCCGCGACCTCTGTTACCGTACCG	GGGCTCTAGAATACCCTTGCAGAT
	17753-17732	17728-17704	17678-17701
vIRF3	GGAGAAGACCA^AGGCCATTTG	TGAGGAGGATCACCCAGCCTTTTGC	CTGCGTGACCGGCACAT
	90952-90942^90847-90838	90815-90791	90773-90789
ORF50	GCAAGATGACAAG^GGTAAGAAGC	CTGTGTGGAAAGCTTCGTCGGCCTC	TGGTAGAGTTGGGCCTTCAGTT
	71601-73613^72572-72581	72592-72616	72645-72624
K3	GCGGGTTGAAGTGTTTCCAT	TCGGCCGACATCACCAGAGTGTG	TTTCCTGAAGCTCGATCTCCTCTA
	19139-19120	19112-19090	19063-19086
K5	TCCACCCGCAGTGTTTAAGC	TGTCTCGAAACACGGCCTGTCAAATG	CGTGCGCGTGCGGTATA
	26368-26349	26335-26310	26283-26299

^ denotes splice junction.