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Batf3 deficiency is not critical for the generation of CD8 α ⁺ dendritic cells

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Abstract

Recently, we have reported that CD8 α ⁺ DCs, rather than CD8⁺ T cells, are involved in the establishment and maintenance of HSV-1 latency in the trigeminal ganglia (TG) of ocularly infected mice. In the current study, we investigated whether similar results can be obtained using Batf3^{-/-} mice that previously were reported to lack CD8 α ⁺ DCs. However, our results demonstrate that Batf3^{-/-} mice, without any known infection, express CD8 α ⁺ DCs. Consequently, due to the presence of CD8 α ⁺ DCs, no differences were detected in the level of HSV-1 latency between Batf3^{-/-} mice compared with wild type control mice.

Keywords

dendritic cells; HSV-1; latency; knockout; compensation

INTRODUCTION

Dendritic cells (DCs) are highly heterogeneous cells and have been classified into several subsets based on their immunophenotype, resident location, and functional differences

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Disclosure Statement

The authors declare that there are no competing financial interests.

(Shortman and Naik, 2007, Belz and Nutt, 2012). In general, DCs are divided into two main groups: conventional (cDCs) and plasmacytoid (pDCs) (Shortman and Naik, 2007, Belz and Nutt, 2012, Heath and Carbone, 2009). In mouse lymph nodes (LNs), conventional DCs include the CD8 α ⁺ and CD11b⁺ subgroups, the latter subdivided based on CD4⁺ expression (Vremec, et al., 2000, Belz, et al., 2004). Human blood DC antigen 3 (CD141, BDCA3) is reported to be the homologue of mouse CD8 α ⁺ DCs (Jongbloed, et al., 2007).

Recently, we reported that DCs isolated from CD8 α ^{-/-} mice harbor fewer viruses than DCs isolated from wild-type, CD8 β ^{-/-}, or β 2m^{-/-} mice (Mott, et al., 2014). In addition, we demonstrated that CD8 α ⁺ DCs are responsible for enhanced viral latency in ocularly infected mice (Mott, et al., 2014, Mott, et al., 2014, Mott and Ghiasi, 2008, Mott, et al., 2008). In those studies, we showed that HSV-1 infected DCs interfered with DC function, such as inhibiting induction of antiviral responses, leading to higher latency and T cell exhaustion. Similarly, it was shown that the absence of CD8 α ⁺ DCs in mice enhances resistance to the intracellular bacterium *Listeria monocytogenes* (Edelson, et al., 2011). These results are also consistent with reports on the negative role of certain DC populations in control of various viral infections (Engelmayer, et al., 1999, Granelli-Piperno, et al., 1998, Geijtenbeek, et al., 2000, Wu, et al., 2000, Paroli, et al., 2000).

Production of CD8 α ⁺ DCs is regulated by several transcription factors including interferon regulatory factor 8 (Irf8), nuclear factor interleukin 3 regulated (Nfil3), inhibitor of DNA binding 2 (Id2), and basic leucine zipper transcriptional factor ATF-like 3 (Batf3) (Belz and Nutt, 2012, Edelson, et al., 2010, Kashiwada, et al., 2011, Hildner, et al., 2008). Based on these studies, the absence of IRF8, Nfil3, Id2, or Batf3 blocked production of CD8 α ⁺ DCs. However, it was recently shown that CD8 α ⁺ DCs can be induced in the absence of the transcription factors Id2, Nfil3, or Batf3, but not IRF8 (Seillet, et al., 2013). BXH2 mice have a spontaneous point mutation in the IRF8 gene similar to that in IRF8^{-/-} mice that blocks the generation of CD8 α ⁺ DCs (Mott, et al., 2014, Turcotte, et al., 2004, Tailor, et al., 2008).

In our published studies, we have shown that the levels of both HSV-1 glycoprotein B (gB) DNA and latency associated transcript (LAT) RNA were significantly lower in latently infected mice that did not produce CD8 α ⁺ DCs compared with mice that produced CD8 α ⁺ DCs (Mott, et al., 2014). In the present study, we examined whether Batf3^{-/-} mice, which were reported to lack CD8 α ⁺ DCs (Hildner, et al., 2008), may have lower HSV-1 latency, as we reported for BXH2 mice (Mott, et al., 2014). In contrast to BXH2, Batf3^{-/-} mice produced CD8 α ⁺ DCs and had similar levels of HSV-1 latency as WT control mice. Therefore, in the absence of any known infection, our results point to the lack of a key role for Batf3 in generation of CD8 α ⁺ DCs. Thus, among the known transcription factors for the development of CD8 α ⁺ DCs, only IRF-8 is essential for generation of CD8 α ⁺ DCs (Mott, et al., 2014, Turcotte, et al., 2004, Tailor, et al., 2008, Fung-Leung, et al., 1991).

Materials and Methods

Virus and mice

Plaque-purified virulent HSV-1 strain McKrae was grown in rabbit skin (RS) cell monolayers in minimal essential medium (MEM) containing 5% fetal calf serum (FCS) as we described previously (Osorio and Ghiasi, 2003). WT C57BL/6 and C57BL/6-Batf3^{-/-} mice were purchased from The Jackson Laboratories and were bred in-house. Mice were housed in static microisolator cages in negatively pressurized isolation cubicles.

Ocular infection

Mice were infected ocularly with 2×10^5 PFU per eye of HSV-1 strain McKrae in 2 μ l of tissue culture medium and administered as an eye drop without prior corneal scarification.

Confocal microscopy and image analysis of BM-derived DCs cultures

Six week-old mice were used as a source of bone marrow (BM) for the generation of mouse dendritic cells (DCs) in culture as we described previously (Mott, et al., 2009). DCs isolated from WT and Batf3^{-/-} strains of mice were grown on Lab-Tex chamber slides (Sigma-Aldrich, St. Louise, MO) as we described previously (Mott, et al., 2014). Briefly, cells were fixed by incubating slides in methanol for 10 min followed by acetone for 5 min at -20°C . Afterwards, slides were rinsed three times for 5 min each at ambient temperature in PBS containing 0.05% v/v Tween-20 (PBS-T). Slides were then blocked for 30 min at ambient temperature in PBS-T containing 1% w/v BSA. Rat anti-CD8 α (clone 53-6.7, eBioscience, San Diego, CA), rat anti-CD4 (clone Gk1.5, eBioscience), rat anti-CD8 α (clone YTS156.7.7, BioLegend), and hamster anti-CD11c (clone HL3, BD Biosciences) were used for IHC. Immunostaining was done using CD11c/CD4, CD11c /CD8 α , or CD11c /CD8 β antibody combinations and staining for 1 h at 25°C . After three rinses for 5 min each in PBS, slides were incubated for 1 h at 25°C with secondary antibodies labeled with anti-hamster FITC or anti-Rat TRITC (Invitrogen). Slides were washed three times with PBS, air-dried and mounted with Prolong Gold DAPI mounting medium (Invitrogen).

Images were captured at 1024×1024 pixels (original magnification = $20\times$) in independent fluorescence channels using a Nikon C1 eclipse inverted confocal microscope.

FACS analyses

Single cell suspension from wt C57BL/6 or Batf3^{-/-} mouse was made by chopping the spleen into small pieces and incubating in 200 U/ml collagenase D (Worthington Biochemical, Lakewood, NJ) for 45 minutes at 37°C followed by filtering through 40 μ m cell strainer (BD biosciences, San Jose, CA) and washing twice in PBS + 1% BSA. 2×10^6 cells were taken for each test. Cells were then stained with APC-eFluor[®] 780-anti mouse CD11c, APC-anti mouse CD8 β and PE/Cy7-anti mouse CD8 α (all from eBioscience, San Diego, CA) in PBS + 1% BSA containing 1 μ g/ml anti mouse FC-receptor block antibody (BioXCell, West Lebanon, NH). Stained cells were washed twice in PBS + 1% BSA then 5×10^5 cells were acquired on BD FACSCANTO-II (BD biosciences) and results were analyzed using FlowJo software (TreeStar, Ashland, OR).

Genotyping of Batf3^{-/-} mouse

Half cm sections of WT and Batf3^{-/-} mouse tail were processed using the DirectPCR lysis reagent (Cat # 101-T, ViagenBiotech, Los Angeles, CA) as per manufacturer protocol. Briefly, tail pieces were added to approximately 200 ul of lysis reagent containing freshly prepared 0.2-0.4 mg/ml Proteinase K (Cat # 501-PK, ViagenBiotech, Los Angeles, CA) and incubated for 5-6 hours at 55 °C. Following incubation, crude lysates were incubated at 85°C for 45 minutes and 1ul of lysate was used for PCR reactions. Standard PCR was carried out using Ready PCR Mix (Cat # N806, Amresco Corporation, Solon, OH) and the Batf3 Fast MCA-Master Protocol- version 2, September 2011- Stock number 013755 (The Jackson Laboratory, Sacramento, CA). Briefly, 1 ul of crude DNA lysate was amplified at the following conditions: 94°C 3min (1 cycle); 94°C 30sec, 59°C 60sec, 72°C 60sec (35 Cycles); 72°C 2min. Primers used for the analysis were as follows: Primer 11387-Common Forward- 5'-TGC TAT GCA CAA ACC ACA AAC C- 3'; Primer 11388- Wild Type Reverse- 5'-GTT GTG AGT CGA AAC CAC GC-3'; Primer 11390- Mutant Reverse- 5'-GAT ACA GGC TGC TGA TGA TCT GAG-3'. The expected PCR product sizes identified the following mouse genotype: Mutant = 600bp; Heterozygote = 600bp and 288bp; and Wild type = 288bp.

RNA and DNA extraction from TG of latently infected mice

DNA was isolated from homogenized latently infected individual TG using the commercially available DNeasy Blood & Tissue Kit (Qiagen, Stanford, CA) according to the manufacturer's instructions. PCR analyses were done using gB specific primers (Forward - 5'-AACGCGACGCACATCAAG-3'; Reverse - 5'-CTGGTACGCGATCAGAAAGC-3'; and Probe - 5'-FAM-CAGCCGCAGTACTACC-3'). The amplicon length for this primer set was 72 bp. Relative copy numbers for gB DNA were calculated using standard curves generated from the plasmid pAc-gB1 (Ghiasi, et al., 1992). In all experiments, GAPDH was used for normalization of transcripts.

RNA was extracted from latent TG or infected DCs as we have described previously (Mott, et al., 2009, Mott, et al., 2007, Mott, et al., 2007). Following RNA extraction, 1 µg of total RNA was reverse-transcribed using random hexamer primers and Murine Leukemia Virus (MuLV) Reverse Transcriptase from the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), in accordance with the manufacturer's recommendations. The levels of various transcripts were evaluated using commercially available TaqMan Gene Expression Assays (Applied Biosystems) with optimized primer and probe concentrations. Primer-probe sets consisted of two unlabeled PCR primers and the FAMTM dye-labeled TaqMan MGB probe formulated into a single mixture. Additionally, all cellular amplicons included an intron-exon junction to eliminate signal from genomic DNA contamination. PCR analyses were done using LAT specific primers (forward primer 5'-GGGTGGGCTCGTGTTACAG-3'; reverse primer, 5'-GGACGGGTAAGTAACAGAGTCTCTA-3'; and probe, 5'-FAMACACCAGCCCGTTCTTT-3'- Amplicon Length =81 bp, corresponding to LAT nts 119553-119634. As an internal control, a set of GAPDH primers from Applied Biosystems (ASSAY I.D. m999999.15_G1 - Amplicon Length = 107 bp) was used.

Quantitative real-time PCR (qRT-PCR) was performed as we described previously (Mott, et al., 2007, Mott, et al., 2007). Real-time PCR was performed in triplicate for each tissue sample. The threshold cycle (Ct) values, which represents the PCR cycle at which there is a noticeable increase in the reporter fluorescence above baseline, were determined using SDS 2.2 Software. GAPDH transcript was used for normalization purposes.

Statistical analysis

Student's *t* tests were performed using the computer program InStat (GraphPad, San Diego). Results were considered statistically significant when the *P* value was < 0.05.

RESULTS and DISCUSSION

In our published study we have shown that BHX2 and CD8 $\alpha^{-/-}$ mice did not produce CD8 α^{+} DCs, while WT, CD8 $\beta^{-/-}$, and $\beta_2m^{-/-}$ mice produced CD8 α^{+} DCs (Mott, et al., 2014). We have also reported that the absence of CD8 α^{+} DCs was associated with lower HSV-1 latency and reactivation compared with mice that produce CD8 α^{+} DCs (Mott, et al., 2014, Mott, et al., 2014). Therefore, to confirm our recent observation that deficiency of CD8 α^{+} DCs leads to lower latency in ocularly infected mice, we used Batf3 $^{-/-}$ mice that were reported previously to lack CD8 α^{+} DCs (Hildner, et al., 2008). We first set out to confirm that Batf3 $^{-/-}$ mice, similar to BHX2 and CD8 $\alpha^{-/-}$ mice, are unable to produce CD8 α^{+} DCs. BM-derived DCs were isolated from mice that we received upon arrival from the Jackson Laboratory (WT or Batf3 $^{-/-}$ _{JAX}) or from their offspring that we bred in-house (Batf3 $^{-/-}$ _{InHouse}). Cells were stained with various combinations of CD11c, CD8 α , CD8 β , or CD4 antibody. Immunohistochemistry (IHC) results for DCs isolated from Jackson mice are shown in Figure 1. CD8 α^{+} CD11c $^{+}$ or CD8 β^{+} CD11c $^{+}$ expression was not detected on DCs isolated from Batf3 $^{-/-}$ _{JAX} mice, while CD4 $^{+}$ CD11c $^{+}$ cells were present (Figure 1, Batf3 $^{-/-}$ _{JAX} DCs). In contrast, BM-derived DCs from our in-house breeding colony expressed both CD8 α^{+} CD11c $^{+}$ and CD8 β^{+} CD11c $^{+}$ cells (Figure 2). Similar to the Batf3 $^{-/-}$ _{JAX} result (Figure 1, CD4), expression of CD4 $^{+}$ CD11c $^{+}$ cells in BM-derived DCs from in-house breeding was not affected (Figure 2). The expression level of CD8 α^{+} CD11c $^{+}$ in BM-derived DCs from Batf3 $^{-/-}$ _{InHouse} mice was similar to the results for WT mice. Taken together, these results suggest that due to some environmental factor, mice that lack the *Batf3* gene reprogram hematopoietic stem cells to allow for production of CD8 α^{+} DCs after we start breeding them in-house.

The IHC results described above show expression of CD8 α^{+} in in-house-derived DCs compared with Jackson -derived DCs. Therefore, it was of interest to determine if similar changes could be detected in the spleens of Jackson versus in-house mice. Single cell suspensions of spleen cells were prepared as described in Materials and Methods. Cells were stained with anti-CD3, anti-CD11c, and anti-CD8 antibodies. Cells were gated for expression of CD11c $^{+}$ CD8 α^{+} and the FACS data are shown in Figure 3. Similar to our IHC results for BM-derived DCs, 0.09% of DCs in the spleens of Jackson mice were CD11c $^{+}$ CD8 α^{+} , while this increased to 0.60% in the spleens of in-house mice (Figure 3). This was similar to the 0.54% level of CD11c $^{+}$ CD8 α^{+} expression in WT mice (Figure 3). Thus, these results further support our in vitro data and suggest that the inability to produce

CD8 α^+ DC in *Batf3*-deficient mice is overcome by another factor upon in-house breeding, possibly due to changes in animal housing conditions.

Previously, it was shown that mice of C57BL/6 genetic background at steady-state express low levels of CD8 α^+ DCs in their spleens and lymph nodes (Edelson, et al., 2011, Jackson, et al., 2011). Similarly, in this study we detected CD8 α^+ DCs in spleens and BM-derived DCs of mice with the same genetic background. However, the expression level of CD8 α^+ DCs in the in-house *Batf3*^{-/-} mice was similar to that of WT mice. It was recently shown that IL-12 administration or infection with *T. gondii*, *L. monocytogenes*, and *Mycobacterium tuberculosis* restored CD8 α^+ DCs production in *Batf3*^{-/-} mice (Tussiwand, et al., 2012). The authors concluded that *Batf* and *Batf2* compensate for the absence of *Batf3* gene during infection with intracellular pathogens, and this is mediated by IL-12 and IFN- γ . Basic leucine zipper transcription factor ATF-like (*Batf*, *Batf2* and *Batf3*) comprise the *Batf* family, which is a subgroup of the larger family of basic leucine zipper (bZIP) transcription factors (Finn, et al., 2010). The expression of *Batf* and *Batf3* is restricted to the haematopoietic system (Hildner, et al., 2008, Williams, et al., 2001), while *Batf2* is expressed in both haematopoietic and non-haematopoietic tissues (Su, et al., 2008). *Batf* is required for the differentiation of interleukin 17 (IL-17)-producing helper T cells (T_H17 cells) (Schraml, et al., 2009, Ise, et al., 2011), promotes effector CD8 T-cell differentiation (Kuroda, et al., 2011), and antibody production (Betz, et al., 2010). *Batf2* exerts cancer-selective growth-inhibitory effects (Su, et al., 2008) and *Batf2*^{-/-} mice had normal development of NK, T and B cells, pDCs, neutrophils, resting cDCs, and peritoneal, liver and lung macrophages (Tussiwand, et al., 2012). *Batf3* was originally reported to be required for development of the CD8 α^+ lymphoid-resident DC subset and of the related CD103⁺ peripheral DC subset, and produce IL-12 in response to pathogens (Hildner, et al., 2008, Williams, et al., 2001, Mashayekhi, et al., 2011). Thus, these studies suggest that *Batf*, *Batf2* and *Batf3* genes each have their unique and non-overlapping function. However, when using *Batf*^{-/-}*Batf3*^{-/-} and *Batf2*^{-/-}*Batf3*^{-/-} mice a reduction of CD8 α^+ cDC expansion was shown following IL-12 treatment compared with similarly treated *Batf3*^{-/-} mice (Tussiwand, et al., 2012). The authors concluded that expression of either *Batf* or *Batf2* can compensate for *Batf3* in the expansion of the CD8 α^+ cDC population. Although both *Batf*^{-/-} and *Batf2*^{-/-} mice had no apparent DCs defect (Tussiwand, et al., 2012). However, the involvement of other factors to compensate for the absence of *Batf3* in the absence of any infection or IL-12 injection can not be ruled out. Moreover, our in-house mice are certified pathogen-free and were not exposed to any type of infection. Thus, it is possible that, in contrast to the reported study by Tussiwand et al (Tussiwand, et al., 2012), changes in microflora of in-house mice that are independent of any type of infection contribute to the appearance of CD8 α^+ DCs in *Batf3*^{-/-} mice. It is widely understood that intestinal microflora can profoundly influence the homeostasis of the host immune system (Iliev, et al., 2012, Tanoue, et al., 2010, Jarchum and Pamer, 2011, Chinen and Rudensky, 2012). In addition, commensal microbes can affect immune cell progenitors (Gollwitzer, et al., 2014, Trompette, et al., 2014, Khosravi, et al., 2014). Consequently, the changes in microflora without any infection may be the reason that the Jackson mice do not express CD8 α^+ DCs, while our in-house mice do.

Both IHC and FACS data described above have shown that CD8 α ⁺ DCs are produced at WT levels in Batf3^{-/-}_{InHouse} but not in Batf3^{-/-}_{JAX} mice. To confirm the absence of *Batf3* gene in both JAX and in-house Batf3^{-/-} mice, qPCR was performed on mice genomic DNA as described in Materials and Methods. As expected, DNA isolated from both Jackson and in-house Batf3^{-/-} mice produced a band of 600 bp, while DNA isolated from WT mice produced a band of 288 bp (Fig. 4). Thus, both JAX and in-house Batf3^{-/-} mice lack the *Batf3* gene and the observed differences are not due to genetic reversion.

Finally, to determine if the absence of *Batf3* gene, despite the presence of CD8 α ⁺ DCs, alters latency in infected mice, WT and Batf3^{-/-}_{InHouse} mice were ocularly infected with HSV-1, as described in Materials and Methods. No differences in virus replication in the eye, eye disease, or survival were detected between WT and Batf3-deficient mice (not shown). Individual TG from surviving mice were isolated on day 28 post-infection (PI), and total DNA was prepared. TaqMan PCR was performed as described in Materials and Methods to quantitate viral gB DNA as a measure of HSV-1 genome copies and hence, latency. The amount of viral DNA during latency in WT mice was similar to that of Batf3-deficient mice (Figure 5A; WT vs. Batf3^{-/-}, p>0.05). This suggests that the lack of Batf3 does not impact viral latency, consistent with the normal presence of CD8 α ⁺ DCs in these mice (Mott, et al., 2014). During HSV-1 neuronal latency, only the LAT region is consistently expressed at high levels (Rock, et al., 1987, Dobson, et al., 1990). We next sought to confirm our gB DNA latency results by quantitating LAT expression levels. On day 28, TG from surviving mice were harvested, and expression of LAT was determined by TaqMan RT-PCR from total RNA isolated from individual TG. In agreement with the gB DNA results (Figure 5A), the amount of LAT RNA detected in the TG of Batf3-deficient mice was similar to that of WT mice (Figure 5B, p>0.05). These results demonstrate that the absence of Batf3 did not reduce HSV-1 latency due to the presence of CD8 α ⁺ DCs.

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Figure 1. IHC of DCs isolated from *Batf3*^{-/-} JAX mice

BM-derived DCs from WT and *Batf3*^{-/-} mice following arrival from The Jackson Laboratory were isolated and grown on Lab-Tex chamber slides. At 24 hr post culture, DCs were fixed and stained with anti-CD11c/anti-CD4, CD11c/anti-CD8α, or CD11c/anti-CD8β antibodies, followed by incubation with relevant secondary antibody to each primary antibody as described in Materials and Methods. DAPI is shown as a nuclear counter-stain. Upper panels: from left to right DAPI, CD11c (FITC), CD4 (TRITC), Merge; middle panels: from left to right DAPI, CD11c (FITC), CD8α (TRITC), Merge; and bottom panels: from left to right DAPI, CD11c (FITC), CD8β (TRITC), Merge.

Figure 2. IHC of DCs isolated from $Batf3^{-/-}$ in-house mice

BM-derived DCs from WT and $Batf3^{-/-}$ mice bred in-house were isolated and grown on Lab-Tex chamber slides and processed as described in the legend of Figure 1 above. DAPI is shown as a nuclear counter-stain. Upper panels: from left to right DAPI, CD11c (FITC), CD4 (TRITC), Merge; middle panels: from left to right DAPI, CD11c (FITC), CD8 α (TRITC), Merge; and bottom panels: from left to right DAPI, CD11c (FITC), CD8 β (TRITC), Merge.

Figure 3. CD8α⁺ expression was altered in *Batf3*^{-/-} offspring without infection

Spleens from *Batf3*^{-/-} JAX and their in-house offspring, as well as from WT mice, were isolated, single cell suspensions were prepared, and subsequently stained with anti-CD11c and anti-CD8 antibodies and gated for expression of CD11c and CD8α. A minimum of 10⁵ events were acquired on a gate including viable cells. Numbers in each quadrant indicate the percentage of single or double-positive cells for each treatment condition.

Fig. 4. Confirmation of the absence of Batf3 in JAX and in-house knockout mice

Tail DNA from WT and Batf3^{-/-} mice was isolated and PCR was performed using the primers as mentioned in Materials and Methods. Panels: (Left) Shown is a representative gel scan from WT and JAX mice demonstrating a band of 600 bp in Batf3^{-/-} JAX mice and a band of 288 bp in WT mice; and (Right) Shown is a representative gel scan from WT and Batf3^{-/-} in-house mice demonstrating a band of 600 bp in in-house mice and a band of 288 bp in WT mouse.

Figure 5. Quantitation of gB DNA and LAT RNA in trigeminal ganglia of HSV-1 latently-infected mice

Wild-type (WT) C57BL/6 or Batf3^{-/-} mice were ocularly-infected with HSV-1 strain McKrae. On day 28 PI, TG were harvested from latently infected mice. Quantitative PCR and RT-PCR was performed on each individual mouse TG. In each experiment, an estimated relative copy number of the HSV-1 gB (for viral DNA) and LAT (for viral RNA) were calculated using standard curves generated from pGem-gB1 and pGem5317, respectively. Briefly, DNA template was serially diluted 10-fold such that 5 μl contained from 10³ to 10¹¹ copies of gB, then subjected to TaqMan PCR with the same set of primers. By comparing the normalized threshold cycle of each sample to the threshold cycle of the standard, the copy number for each reaction was determined. GAPDH expression was used to normalize

the relative expression of viral (gB) DNA and LAT RNA in the TG. Each data point represents the mean $\pm$ SEM from 10 TG. Panels: A) gB DNA; and B) LAT RNA.