Human herpesvirus 6A infection and immunopathogenesis in humanized Rag2−/−γc−/− Mice

Running title: HHV-6A infection in humanized mice

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Abstract

Although serious human diseases have been correlated to human herpesvirus 6A (HHV-6A) and human herpesvirus 6B (HHV-6B), the lack of animal models has prevented studies which would more definitively link these viral infections to disease. HHV-6A and HHV-6B have recently been classified as two distinct viruses and in this study we focus specifically on developing an in vivo model for HHV-6A. Here we show that Rag2<sup>−/−</sup>γ<sub>c</sub>-<sup>−/−</sup> mice humanized with cord blood-derived human hematopoietic stem cells produce human T cells that express the major HHV-6A receptor, CD46. Both cell-associated and cell-free viral transmission of HHV-6A into the peritoneal cavity resulted in detectable viral DNA in at least one of the samples (blood, bone marrow, etc.) analyzed in nearly all engrafted mice. Organs and cells positive for HHV-6A DNA were the plasma and cellular blood fractions, bone marrow, lymph node, and thymic samples whereas control mice had undetectable viral DNA. We also noted viral pathogenic effects on certain T cell populations. Specific thymocyte populations were significantly modified in cell-associated infected humanized mice, including CD3<sup>−</sup>CD4<sup>+</sup>CD8<sup>−</sup> and CD3<sup>+</sup>CD4<sup>−</sup> cells. In addition, we detected significantly increased proportions of CD4<sup>+</sup>CD8<sup>+</sup> cells in the blood of cell-free infected animals. These findings provide additional evidence that HHV-6A may play a role in human immunodeficiencies. These results indicate that humanized mice can be used to study *in vivo* infection and replication of HHV-6A, including aspects of viral pathogenesis.
Introduction

Human herpesvirus 6 (HHV-6) is a member of the β-herpesvirus subfamily and was identified in 1986. (1) Recently this virus has been reclassified as two distinct variants, HHV-6A and HHV-6B based upon differences in tropism, disease, and epidemiology. These two variants have an overall nucleotide identity of 90% (2, 3) and serological assays to differentiate the variants are in development. (4) The main cellular receptor for HHV-6A is CD46, which is expressed on all nucleated cells. (5) CD134 has recently been identified as a cellular receptor for HHV-6B. (6) While HHV-6B infection is ubiquitous in humans and is known to cause roseola infantum (7), the prevalence of HHV-6A and its role in human disease is poorly understood. HHV-6 has been implicated in diseases including multiple sclerosis (8-10), encephalitis, graft-versus-host disease (11, 12), other clinical complications of solid organ transplant and hematopoietic stem-cell transplants (13, 14), drug induced hypersensitivity syndrome (15, 16), malignancies, myocarditis, and cardiomyopathy (17, 18). HHV-6A has an impact upon human T cell populations (19), and can enhance HIV-1 replication (20). HHV-6A infects helper T cells as does HIV-1, and HHV-6A has been suggested as a potential co-factor in AIDS progression. (18, 20-22)

A variety of animals models have been explored for HHV-6 studies, but with limited success. Early reports indicated that HHV-6A was able to replicate in T cells isolated from chimpanzees (23) and pigtailed macaques. (24) More recent reports have shown that non-human primate (NHP) models exhibit signs of disease following infection with HHV-6. Leibovitch et al. recently showed that common marmosets can be infected with HHV-6A, accompanied by
neurological symptoms.(25) Lusso et al. demonstrated that HHV-6A replicates in vivo in pigtailed macaques, and that co-infection of macaques with HHV-6A and Simian Immunodeficiency Virus resulted in faster depletion of CD4+ T cells.(22) The requirement for specialized facilities and the expenses involved in NHP research have been detrimental to further studies.

A small animal model of HHV-6 infection would allow for further investigation of viral pathogenesis without the costs and facilities required for NHP research. The viral target cells in humanized mice are human immune cells and hence viral infection in humanized mice may be more reflective of human infection due to differences in human and NHP genetics. Additionally, humanized mice can be infected with HIV-1 as opposed to the genetically distinct SIV isolates used in NHP models. Humanized mice infected with HIV-1 manifest symptoms of AIDS (26) for studies of HHV-6A as a co-factor in AIDS progression.

Here we report on the use of a newer generation of humanized mice to study HHV-6A replication and pathogenesis in vivo. Rag2⁻/⁻γc⁻/⁻ mice (RAG-hu mice) are engrafted with human hematopoietic stem cells (HSCs) and undergo multi-lineage hematopoiesis to produce a variety of human blood cell types which are dispersed throughout the lymphoid and non-lymphoid organs. These mice (and other similar HSC-humanized mouse models) have been shown to support replication and viral pathogenesis after challenge with the herpesviruses EBV(27-30), KSHV(31, 32), and hCMV (33). Here, RAG-hu mice were challenged with either cell-free or cell-associated recombinant HHV-6A expressing green fluorescent protein (GFP). Our findings show that viral DNA is detectable in blood and lymphoid organs for up to 8 weeks after infection. Viral DNA was detected in the plasma, blood cells, thymus, lymph node, and bone marrow, although no single mouse tested positive for viral DNA in all of these compartments, 11
out of 12 mice were positive in at least one. Specific thymocyte populations were found to be significantly modified in animals infected for longer periods (and via cell-associated transmission); while animals infected with cell-free virus showed a significant increase in CD4⁺CD8⁺ cells in blood. These findings suggest that humanized mice represent a new *in vivo* model to study HHV-6A replication and immunopathogenesis.
Materials and Methods

Cells

Human cord blood samples were obtained with permission from the University of Colorado Cord Blood Bank. The Institutional Review Board does not require a protocol for human cord blood because samples are shipped without patient identifiers. HSCs were purified from human cord blood based upon the CD34 marker using the EasySep Human Cord Blood CD34 positive selection kit (StemCell Technologies). Cells were cultured for two days in IMDM (Invitrogen) supplemented with 10% FCS and 10 ng/ml each of human IL-3, IL-6, and SCF (R&D Systems).

Virus propagation

BAC-derived HHV-6A, strain U1102, was previously engineered to express GFP (34). BAC-derived HHV-6A DNA was isolated from overnight E. coli cultures grown at 32°C in LB containing chloramphenicol (15μg/ml) and purified using NucleoBond PC 100 columns (Clontech) per the manufacturer’s protocols. HHV-6A BAC DNA (5μg) and 1μg of the human cytomegalovirus pp71-expressing plasmid pCGN1-pp71 (35) were transfected into 5×10^6 Jhan cells with transfection reagent “V” utilizing a Nucleofector, (Lonza AG) per the manufacturer’s protocols. After transfection the cells were maintained in 3mL RMPI media containing 8% Fetal Bovine Serum (Sigma) and supplemented with 100 U/ml each of penicillin and streptomycin. After 5-7 days the media was changed and supplemented with 20ng/mL TPA (Sigma) and 3mM Na-butyrate (Sigma) for 24 hours. Cells were washed 3x with PBS to remove the TPA and Na-butyrate and co-cultured with an equal number of HSB-2 cells that were pre-stimulated for 24
hours with 2pg/ml IL-2 (Sigma) and 5ng/ml PHA (Sigma). Fresh pre-stimulated HSB2 cells are
added every 4-6 days to allow accumulation of the virus by cell-to-cell spread.

To isolate virus, the cultures were pelleted by low-speed centrifugation and the
supernatant was reserved. Infected cells were resuspended in 10 ml of media and sonicated to
release virus from infected cells. The media was then cleared of cellular debris, and the
supernatant was added to the reserved media. Virus was then purified by ultracentrifugation
through a 20% sorbitol cushion in a SW28 rotor for 90 minutes at 53,000 x g. The resulting
pellet was resuspended in media supplemented with 1.5% BSA and aliquots were stored at –
80°C following snap-freeze in liquid nitrogen.

**Titering of Virus**

Titers of HHV-6A were calculated using standard TCID50 assays (36). Briefly, Jjhan cells
were plated into a 96-well plate at 1×10^5 cells per well and incubated overnight at 37°C. Aliquots
of HHV-6A were thawed at 37°C and briefly sonicated. The stock was serially diluted in 10-fold
increments and used to inoculate Jjhan cells. The cultures were incubated in a 37°C incubator
with 5% CO₂ for 10-14 days. GFP positive wells were scored to determine the titer of the stock.

**Animals**

Balb/c-Rag2−/−γc−/− mice were humanized by engraftment with CD34⁺ human HSCs
purified from human umbilical cord blood as described previously.(37) Mice were maintained in
the specific pathogen-free room at the Brigham Young University Central Animal Care Facility.
These studies have been reviewed and approved by the Institutional Animal Use and Care
Committee (Protocol 120101). Briefly, 1-5 day old mice were conditioned by irradiating with
350 rads and then injected intrahepatically with 2-5×10⁵ human CD34⁺ cells. Mice were
screened for human cell engraftment at 8 weeks post-engraftment. Peripheral blood was collected by tail bleed and stained with antibodies specific to either human or mouse CD45. FACS analysis was performed to determine percent peripheral blood engraftment of human cells.\Textsuperscript{(38, 39)}

**Preparation of carrier cells for viral transmission to humanized mice**

\[1 \times 10^6\] fresh CD34 depleted cord blood mononuclear cells were cultured in basal medium (RPMI 1640 + 10% FCS + 1x Pen/Strep) and were stimulated with PHA (20 \(\mu\)g/ml) for 48 hours followed by IL-2 (100 units/ml) for 10 days. We chose to use these cells because 1) they are routinely used for HHV-6A infections and 2) they are readily available in our lab. DNA extraction and quantitative polymerase chain reaction (Q-PCR) were performed on a portion of cells to verify lack of endogenous HHV-6A. \(1.7 \times 10^6\) cells were infected with \(3.3 \times 10^5\) infectious units (i.u.) of a recombinant strain of HHV-6A expressing GFP under the CMV IE promoter, and in the U1102 strain background\Textsuperscript{(34)} (hereafter referred to as HHV-6A) + 5 \(\mu\)g/ml polybrene (Sigma) and mock-infected cells were resuspended in basal medium with polybrene. Samples were infected for 2 hours, shaken every 30 min. Final MOI was 0.02. After incubation, cells were resuspended in 3 ml basal medium and plated in a 6-well plate. IL-2 was added and cells were incubated for 48 hours. FACS analysis was performed on infected and mock-infected cells to detect and quantify HHV-6A-infected cells prior to mouse infection. GFP was used to identify infected cells, and samples were stained with anti-human CD3, CD4, and CD8 antibodies (see below) to characterize infected cell types.

**HHV-6A transmission to humanized mice**
In the cell-associated viral transmission study, 1×10^5 cells (of which ~20% were GFP+) in 100 µl serum-free RPMI 1640 were injected intraperitoneally (i.p) into mice. Uninfected mice were injected similarly with uninfected cells in the same medium as infected mice. In the cell-free viral transmission study, cell-free HHV-6A-GFP was thawed and immediately diluted in RPMI 1640 (no serum or antibiotics). 100 µl of cell-free virus (4.3×10^5 i.u./mouse) was injected i.p. into mice.

Measurement of blood viral load

Blood was collected by tail bleed for 6 weeks. 70 µl of whole blood was collected per time point and blood was usually centrifuged to separate cellular and plasma fractions. DNA was then extracted with the QIAamp DNA Blood Mini Kit (Qiagen). Q-PCR was performed using an Applied Biosystems StepOne machine to detect and quantify presence of viral genomes using a published assay. (40) 10-fold serial dilutions of a plasmid containing the target HHV-6A sequence were used as copy number standards, and the sensitivity of the assay was previously reported to be 10 DNA copies. (40) The limit of detection of the assay was 1,000 normalized copies in plasma/ml or 400 copies in bone marrow, lymph node, thymus, and spleen (see below).

Organ collection and measurement of viral load in organs

In the cell-associated study, mice were sacrificed (time points ranging from 6.5 to 9.5 weeks post-infection, see Table 1) and lymphoid organs collected (thymus, bone marrow, lymph nodes and spleen). Bone marrow was extracted from both femurs. The thymus was divided in half for Q-PCR or FACS analysis in one infected and one mock-infected animal. Subsequently, single cell suspensions were made and divided in half in order to perform both FACS and Q-PCR analysis. For Q-PCR, DNA was extracted using the QIAamp DNA blood mini kit and
analyzed by Q-PCR as described above. Similar methods were used for organ collection in the cell-free viral transmission study except mice were sacrificed at 1 week post-infection (p.i.).

**FACS analysis**

Anti-human CD45 (eBioscience) and anti-mouse CD45 (eBioscience) were used for screening mice pre-infection to determine percent engraftment. Anti-human CD3 (BioLegend), anti-human CD4 (eBioscience), anti-human CD8 (eBioscience) and anti-human CD46 (eBioscience) were used in FACS analysis of regular tail bleeds and on the harvested organs. Samples were run on a BD FACSCanto flow cytometer and analyzed with Summit v4.3 software.

**Results**

**RAG-hu mice produce cells that express the HHV-6A receptor**

RAG-hu mice were engrafted with human CD34+ hematopoietic stem cells isolated from cord blood as described previously (37) and as outlined in Methods. Mice were screened for human cell engraftment at 8 weeks post reconstitution by FACS analysis of peripheral blood for the pan-leukocyte markers hCD45 and mCD45.

CD46 is a known receptor involved in HHV-6A entry (5) and serves as an inhibitor of complement-mediated cell lysis. CD46 is thought to be expressed in all nucleated human cells, but is only expressed in murine testis (41) which may explain murine resistance to HHV-6A infection. Thus, we stained cells from RAG-hu mice for the presence of CD46 in order to
determine if this animal model might be useful for HHV-6A research. We found that RAG-hu mice produce human CD46+ cells in the blood, thymus, and bone marrow (Fig.1). CD3+ T cells were CD46+, as well as CD3- cells that were not characterized further.

Infection of RAG-hu mice with HHV-6A

Initial attempts at HHV-6A infection used cell-associated virus because HHV-6 is known to be a highly cell-associated virus (42) plus a recent study using the related hCMV in humanized mice was unable to achieve infection with cell-free virus but was successful using infected fibroblasts as carrier cells.(33) We also attempted cell-free virus transmission with a high titer stock of HHV-6A to determine if this mechanism would also be viable for inoculation of humanized mice. Detection of cell-free transmission is greater evidence for the permissiveness of in vivo infection because in cell-associated transmission the input virus may subsequently be detected whether transmission to the graft takes place or not.

In the cell-associated transmission study mice were divided into 5 groups: 1) non-humanized Rag2-/-γc-/- mice, never irradiated, inoculated with infected cells, 2) 0% engrafted Rag2-/-γc-/- mice, irradiated, inoculated with infected cells, 3) >30% engrafted Rag2-/-γc-/- mice, inoculated with infected cells, 4) >30% engrafted Rag2-/-γc-/- mice, inoculated with uninfected cells, and 5) engrafted Rag2-/-γc-/- mice, not inoculated, uninfected (Table 1). Animals with >30% peripheral blood engraftment (defined as (hCD45+ cells)/(hCD45+ cells + mCD45+ cells)) were used in order to ensure that the human immune system is sufficient to support viral infection. Group 1 served as a control for determining if HHV-6A could infect and/or persist in non-humanized immunocompromised mice. Group 2 served as a control for determining if HHV-6A could infect mice that had been sub-lethally irradiated (thus becoming further
immunocompromised) and reconstituted but had undetectable engraftment. Group 3 is the experimental group to determine if HHV-6A could infect engrafted RAG-hu mice. Groups 4 and 5 served as uninfected controls. Cells used for viral transmission were not donor matched with cell samples used to engraft, similar to a previous report where successful hCMV transmission was accomplished with allogeneic human fibroblasts in a related humanized mouse model (33). In the cell-free transmission study mice were divided into 2 groups: 1) non-humanized, never irradiated, inoculated with cell-free virus and 2) >30% engrafted, inoculated with cell-free virus. Mice in the >30% groups in both studies ranged from 30-75% engraftment (see Table 1).

All RAG-hu mice were tested for the presence of HHV-6A DNA by Q-PCR prior to experimental infection. This verification was necessary because a low percentage of human cord blood samples (which are used to initially engraft the humanized mice) are contaminated with HHV-6A.(43) All mice tested negative for HHV-6A DNA in whole blood samples analyzed prior to cell-associated HHV-6A inoculation as did PHA+IL-2 stimulated cord blood cells prior to HHV-6A infection (used as carrier cells for transmission).

**Generation of infected cells for use in cell-associated transmission study**

PHA and IL-2 stimulated CD34 depleted cord blood mononuclear cells were infected with HHV-6A or uninfected for 2 hours as described previously in Methods. Cells were cultured for 48 hours p.i. (with green cells present upon visual inspection by fluorescence microscopy at 20 hours p.i. in infected sample; data not shown). We observed an increase in cell size in the infected group as compared to the uninfected group, which is a common cytopathic effect of HHV-6A (data not shown). We also observed a CD3^{low}CD4^+ subgroup in the infected sample (Fig 2D) that was not present in the uninfected sample (Fig 2C), and downregulation of CD3 is
also common upon HHV-6A infection of T cells. (44) Approximately 20% of lymphocytes were GFP+ in the infected sample (Fig 2B) immediately prior to injection into RAG-hu mice with a low background of GFP expression in the uninfected sample (Fig 2A).

**HHV-6A DNA detection in blood and lymphoid organs**

All animals were bled regularly and DNA was extracted for Q-PCR analysis of the viral genome from different blood fractions (plasma, blood cell, or whole blood, as indicated in tables 2 and 3). In addition, animals were sacrificed at various time points (see Table 1) in order to examine various organs for viral genome detection and/or to analyze cellular populations for GFP expression and for depletion or enrichment of specific T cell populations. Viral DNA was detected by Q-PCR in nearly all animals at at least one time point in the >30% engrafted, HHV-6A inoculated groups (both cell-free transmission and cell-associated transmission see tables 2, 3 and 4).

In the cell-free transmission study viral DNA was detected in the bone marrow of all 6 >30% engrafted, HHV-6A inoculated mice while no viral DNA was detectable in the non-humanized mice (n=4; see Table 2). 2 of the >30% engrafted, HHV-6A inoculated mice had detectable viral DNA present in the blood whereas no viral DNA was detected in the blood of infected, unengrafted mice. No viral DNA was detected in thymic (n=3) or splenic tissues (n=6) analyzed from cell-free infected RAG-hu mice. All samples analyzed in the cell-free transmission study were done at the time of sacrifice (1 week post infection).

In the cell-associated transmission study, HHV-6A DNA was detected by Q-PCR in plasma and cellular fractions of blood (Table 3), in the bone marrow, lymph node, and thymus but not in spleen (Table 4). Viral DNA was detected in mice in the >30% engrafted,
HHV-6A inoculated group in the plasma fraction in 4 of 6 mice, in the blood cell fraction in 3 of 6 mice tested, and in 1 of 6 whole blood samples tested. In addition, 2 of 6 mice in this group had detectable viral DNA in the bone marrow and in the lone mouse thymic and lymph node samples tested from this group (see Table 4). We noted that viral DNA was detected mostly in the plasma from weeks 1-5 and that plasma viral load decreases in copy number after week 4 and in frequency of detection after week 5. 4 of 18 samples collected from engrafted humanized infected mice during the first three weeks of the cell-associated infection experiment were positive for viral DNA, while 15 of 38 samples tested from week 4 onward were positive and had generally higher levels of viral DNA. In total, 5 of 6 mice in the >30% engrafted, HHV-6A inoculated group had viral DNA present in blood or organs. Mouse 708 had undetectable viral DNA in blood or lymphoid organs, but this animal was inadvertently inoculated with about half of the volume of infected carrier cells into the subcutaneous space and the other half into the intended intraperitoneal cavity. No viral DNA was detected in any of the three control groups in the cell-associated transmission study.

Detection of HHV-6A-infected cells in vivo via GFP expression

We attempted to detect GFP⁺ cells as an additional way to verify successful infection in both the cell-associated and cell-free infection studies. However, no GFP⁺ cells were detected in the cell-associated study in blood samples collected weeks 1 and 3 post-infection and analyzed by flow cytometry. Additionally, no GFP⁺ cells were detected when lymphoid organs were collected at the time of sacrifice. A single mesenteric lymph node sample (mouse 715) from the cell-free transmission study was found to harbor GFP⁺ cells when animals were sacrificed and analyzed at 1 week post-infection (data not shown). 0.04% of CD4⁺ cells in the lymph node were
GFP⁺, while 0.28% of CD8⁺ cells were GFP⁺. Of the CD4⁺GFP⁺ cells, most were CD3⁻ (95%). Of the GFP⁺ cells detected in this sample, 4% were CD3⁺, 55% were CD4⁺, and 7% were CD8⁺.

**Thymocyte populations are significantly changed in HHV-6A infected RAG-hu mice**

Previous work in humanized mice (SCID-hu thy/liv model) infected with HHV-6A or HHV-6B indicated that these viruses are capable of modifying thymic populations after direct viral inoculation into the thymic graft.(19) We thus analyzed thymic populations taken from RAG-hu mice infected by either cell-associated or cell-free transmission. Animals infected by cell-free transmission had undetectable levels of viral DNA in the thymus at 1 week post-infection (3 of 3 tested; see Table 2), and their thymocyte populations were similar to uninfected animals (data not shown). Thus, we focused our thymocyte analysis on animals infected by the cell-associated transmission route, noting that these animals were also infected for a longer duration. RAG-hu mice infected by cell-associated transmission did exhibit significant shifts in thymic populations (Fig 3). We noted a significant decrease (P=0.05) in CD3 expression on CD4⁺ thymocytes with a mean of 3.9% and 17.9% of thymocytes that were CD3⁻CD4⁻ in infected (n=4) and uninfected (n=6) groups, respectively. This is similar to a previous report indicating that HHV-6 can downregulate CD3 expression.(45) We also detected a significant loss (P=0.04) of intrathymic T progenitor cells (CD3⁻CD4⁻CD8⁻), with a mean of 2.5% in infected mice (n=3) and 11.3% in uninfected mice (n=5) when gating on the CD4⁺ population. When analyzing this same population on a CD3⁻ gate, we again found a significant depletion (P=0.03) with a mean of 2.7% in infected mice (n=3) and 32.2% in uninfected mice (n=5). We also detected a significant (P=0.02) increase in the number of CD3⁻CD4⁻CD8⁺ thymocytes with a mean of 36.6% in infected mice (n=3) and 12.8% in uninfected mice (n=5). The CD3⁻CD4⁻CD8⁺ population appeared to increase in infected animals but the difference was not significant.
and the CD3⁺CD4⁺CD8⁻ population appeared to decrease in infected animals but the difference was not significant (P=0.25).

**Detection of CD4⁺CD8⁺ T cells in HHV-6A-infected RAG-hu blood**

Previous studies have indicated that HHV-6A infection can induce CD4 expression on primary human CD8 T cells in vitro.(46) Thus, we analyzed samples by FACS for the presence of CD4⁺CD8⁺ T cells. We detected significantly increased ratios (P=0.04) of CD3⁺CD4⁺CD8⁺ cells in the blood of cell-free virus infected RAG-hu mice (Fig. 4). This population represented a mean of 8.8% of CD3⁺ T cells, while in uninfected humanized mice these cells were 3.1% of all T cells. CD4⁺CD8⁺ cells are normally rare in human blood, with one report showing an average of 2.91% of CD4⁺CD8⁺ cells in normal human blood (n=10) (47) which is similar to our results in uninfected humanized mouse blood. Other blood T cell populations were not significantly altered in infected samples, including an analysis of CD3⁺CD4⁻CD8⁺, CD3⁺CD4⁺CD8⁻ CD3⁻ CD4⁺CD8⁺, CD3⁻CD4⁺CD8⁻ and CD3⁻CD4⁻CD8⁺ populations.

**Discussion**

Here we have shown that RAG-hu mice are susceptible to infection with HHV-6A by either cell-associated or cell-free transmission. Viral DNA was detected in blood (cellular and plasma fractions), bone marrow, lymph node, and thymic tissues (Tables 2, 3, and 4) although no single mouse tested positive for viral DNA in all of these compartments, as mentioned previously. Following cell-associated transmission, viral DNA was detectable for up to 8 weeks
post-infection indicating a persistent infection. No viral DNA was detected in any of the three control groups in the cell-associated study, indicating that viral transmission from the infected carrier cells to the originally engrafted cells and subsequent replication was successful because no viral DNA was detectable in either blood or lymphoid organs in control mice without an HSC graft. Irradiated but non-engrafted animals were included as a control for the higher murine immunodeficiency of irradiated mice and they also had undetectable viral DNA after transmission. Some animals in the cell-associated transmission study had viral DNA detected at early time points and not later, and some animals had no detectable DNA at early time points but it was detected later. We attribute this to a relatively high limit of detection in the assay because only small blood samples can be obtained from mice. We noted that plasma viral DNA levels peaked at 3-4 weeks post-infection, but levels decreased to near the level of detection in plasma by 5 weeks and only a single animal had detectable viral DNA in the plasma at 6 weeks. Viral DNA was still detected in the cellular fraction of blood at 6 weeks in three animals while plasma viral DNA was found in a single mouse at that time point, potentially indicating a shift from lytic infection (extracellular DNA) to latency (intracellular DNA).

Cell-associated transmission was attempted because a similar previous experiment with hCMV was only successful with this method.(33) However, cell-free transmission was successful in all 6 animals in our study. We also noted a greater tendency to detect viral DNA in the bone marrow of animals infected by cell-free transmission, but it is not clear if that finding is due to a different mode of transmission or to different time points (1w for cell-free and 6.5-9.5w for cell-associated). We also detected a significant increase in CD4^+CD8^+ cells in the blood of cell-free virus infected mice (Fig. 4). This was possibly due to CD4 upregulation in CD8 T cells, which was previously shown in vitro in HHV-6A infected cells.(46) When we correlated Q-PCR
results of blood cells and plasma to the detection of these CD4^+CD8^+ cells there was not a clear trend because one animal (715) had high proportions of these cells and detectable viral DNA in plasma, while other animals also had high proportions of the cells but undetectable viral DNA in either blood fraction. Another animal (711) had low proportions of the cells with only intracellular blood viral DNA. The frequent detection of this effect, combined with relatively rare detection of viral DNA in either blood fraction, indicates that these cells may be uninfected by HHV-6A. These cells largely maintained CD3 expression, which also indicates a lack of infection. It is possible that HHV-6A infection promotes the release of these cells from the thymus because CD4^+CD8^+ cells are rare outside of the thymus. However, we failed to detect viral DNA in the thymus in 3 animals tested and so the promotion of releasing cells from the thymus would likely be conducted from a distal site. Evidence exists that the presence of CD4^+CD8^+ cells in human blood is upregulated following viral infection, including after infection with persistent viruses such as the herpesvirus EBV. (47)

We attempted to use GFP expression from a recombinant virus to further demonstrate successful infection. However, the only animal with detectable GFP^+ cells was mouse 715 from the cell-free transmission group, and those cells were from the mesenteric lymph node. FACS analysis of GFP^+ cells indicated that they were mostly CD3^−CD4^+, which is in accordance with our in vitro results in Fig 2D and previously published data showing a tropism for CD4^+ T cells and a downregulation of CD3 after infection. (45, 48) We later determined that the GFP cassette in this virus is driven by the CMV IE promoter (Y. Mori, personal communication). Since the cell-associated mice were sacrificed at 6.5 to 9.5 weeks p.i. it is possible that the virus was in a latent state at the time of organ collection; this hypothesis is supported by the shift from extracellular to intracellular DNA seen in blood. The activity of the CMV IE promoter in the
context of a latent HHV-6A infection is currently unknown, and if that promoter is inactive
during latency that may explain a lack of GFP+ cells in any of the cell-associated infected mice.
An interesting observation was made that several thymocyte populations were altered in HHV-6A infected animals versus uninfected animals (Fig. 3). These observations lend further support that successful viral transmission occurred, because similar findings have been reported in vitro and in another humanized mouse study. In those studies, CD3 depletion only occurred in infected (not in bystander) cells.(19, 46, 48) Thymocyte depletion was only detected in animals infected by the cell-associated pathway, but these animals were also infected for a longer period. It is possible that virus had not trafficked to the thymus in cell-free infected mice, a finding supported by our Q-PCR data where 0 of 3 of these thymic samples harbored viral DNA (Table 2). We noted significant depletion in the CD3+CD4- and CD3-CD4+CD8- populations in HHV-6A-infected animals. We also noted a significant increase in the CD3+CD4-CD8+ subset, with a marginally significant increase in the CD3+CD4+CD8- population. The significant loss of CD3-CD4+CD8- thymocytes was similarly reported by Gobbi et al. when HHV-6A was directly inoculated into the thymic organoid of SCID-hu thy/liv mice.(19) In contrast to that report, our results show a significant increase in the CD3+CD4+CD8+ subset. These discrepancies may be explained by the use of different virus isolates, with strain GS used in that report and a recombinant isolate based upon strain U1102 used here. In addition, we have used a newer generation of humanized mice with a wider scope of human cell types and a much broader distribution in the mouse, and we inoculated at a site distant from the thymus. Several of these thymocyte populations that were modified by infection in vivo can be explained by a tropism and cytopathogenicity of the virus for CD4+ T cells. Additionally, the tendency of the virus to downregulate CD3 and/or to upregulate CD4 expression can also explain shifting populations
(e.g., CD3^+CD4^- and CD3^+CD4^+CD8^- populations expected to decrease, and CD3^-CD4^-CD8^+ population expected to increase). The CD3^+CD4^-CD8^- population was previously shown to be more infectable with HHV-6A as compared to other thymocyte populations.(19) We have proposed that in the cell-associated study the virus was predominantly latent at the time points that the thymic samples were collected. We are not aware of any studies documenting CD3 downregulation or CD4 upregulation in latently infected primary cells, so it is currently not clear if lytic replication is required for these effects upon host cell gene expression.

We and others have previously shown that RAG-hu mice are also highly susceptible to HIV-1 infection.(26, 38, 49, 50) Our current findings indicate higher proportions of CD4^+ cells in HHV-6A infected animals, similar to those shown by Lusso et al. in vitro where they showed that HHV-6A-infected CD8^+ T cells began to express CD4 and were able to replicate HIV-1.(46) If HHV-6A is able to convert CD8^+ T cells to become infectable by HIV-1 in vivo, then those cells may be depleted by HIV-1 and/or by HHV-6A. Downregulation of CD3, as our results herein have indicated, is expected to cause immunosuppression because CD3 serves as the signaling subunit of the T cell receptor. Hence, T cells could engage the T cell receptor but not be able to respond effectively. Either of these two effects would support the hypothesis that HHV-6A is a co-factor in AIDS progression.(21, 22) Our future directions include plans to perform co-infection studies of HHV-6A and HIV-1 in humanized mice in order to determine if there is a synergistic effect between the two viruses in the progression to AIDS as well as to determine if RAG-hu mice will sustain infection with HHV-6B.
Acknowledgments

We are grateful to Dr. Yasuko Mori (National Institute of Biomedical Innovation, Osaka, Japan) for giving permission to use a recombinant strain of HHV-6A produced in her laboratory. Balb/c-Rag2<sup>-/-</sup>-γc<sup>-/-</sup> mice were generously provided by Dr. Ramesh Akkina at Colorado State University. This work was funded by a Pilot Grant award from the HHV-6 Foundation to BB. This work was partially supported by National Institutes of Health Grant U54 AI057160 to the Midwest Regional Center of Excellence for Biodefense and Emerging Infectious Diseases Research (MRCE) to EM. AT was supported by the Brigham Young University Cancer Research Center and the HHV-6 Foundation.
References


**Figure Legends**

Figure 1. Humanized mice harbor human cells that express the HHV-6A receptor. (A-C) RAG-hu thymocytes are CD46+, including (A) CD3+ T cells and the two major subsets of T cells, (B) CD4+ helper T cells (C) and CD8+ cytotoxic T cells. (D-E) RAG-hu blood also contains CD46+ cells, some of which are also (D) CD3+ and (E) CD4+. (F) The bone marrow also harbors CD46+ cells, but only minimal T cells were detected in this organ.

Figure 2. HHV-6A infected carrier cells used in cell-associated transmission to humanized mice. (A-D) Flow cytometry of (A,C) uninfected and (B,D) HHV-6A infected cells prior to injection into RAG-hu mice. (A,B) Detection of GFP expression. (C,D) Analysis of CD3 and CD4 expression.

Figure 3. Depletion of specific thymocyte populations in HHV-6A-infected mice. RAG-hu mice infected with HHV-6A by cell-associated viral transmission showed modulation of specific thymocyte populations. (A-C) The CD3+CD4- population was depleted, while the CD3-CD4+ was increased in infected animals. Mouse 3095 (uninfected) is shown in A and mouse 3099 (infected) is shown in B. Samples were gated on a lymphocyte gate. N=4 infected N=6 uninfected. (D-F) The CD3+CD4+CD8- and CD3-CD4+CD8- populations were depleted and the CD3+CD4+CD8+ population increased when analysis was gated upon CD4+ cells. Mouse 3098 (uninfected) is shown in D and mouse 3099 (infected) is shown in E. N=3 infected N=5 uninfected. (G-I) The CD3+CD4+CD8+ population expanded and the CD3+CD4+CD8- population...
was reduced when analysis was gated on the CD3⁻ population. Mouse 3098 (uninfected) is shown in G and mouse 3089 (infected) is shown in H. N=3 infected N=5 uninfected. When performing data analysis, the two single positive and the double positive cell populations were normalized to 100%. Mouse 708 was excluded from these analyses because no viral DNA was detected in that mouse at any time point and we concluded that the mouse likely was not successfully infected. Standard error indicated; student’s t test used for statistical analysis. *, p ≤ 0.05.

Figure 4. Detection of CD4⁺CD8⁺ T cells in blood of HHV-6A infected mice. (A-C) Levels of CD4⁺CD8⁺ T cells in blood were quantified by flow cytometry. (A) uninfected mouse, (B) HHV-6A infected mouse 715 (cell-free transmission, and highest amount of CD4⁺CD8⁺ cells). All samples were first gated on CD3. (C) Mean levels of CD4⁺CD8⁺ cells were quantified in cell-free infected mice (n=6), uninfected mice (n=4). Standard error indicated; student’s t test used for statistical analysis. *, p ≤ 0.05.
### Table 1. Characteristics of humanized mice used for HHV-6A infections

<table>
<thead>
<tr>
<th>Mouse #</th>
<th>Injected w/ HHV-6A</th>
<th>Age (months)</th>
<th>Engraftment</th>
<th>Irradiated</th>
<th>Sacrificed (weeks)</th>
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<td>+, CA</td>
<td>2</td>
<td>0%</td>
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</tr>
<tr>
<td>34</td>
<td>+, CA</td>
<td>2</td>
<td>0%</td>
<td>-</td>
<td>6.5</td>
</tr>
<tr>
<td>35</td>
<td>+, CA</td>
<td>2</td>
<td>0%</td>
<td>-</td>
<td>9.5</td>
</tr>
<tr>
<td>36</td>
<td>+, CA</td>
<td>2</td>
<td>0%</td>
<td>-</td>
<td>9.5</td>
</tr>
<tr>
<td>685</td>
<td>+, CA</td>
<td>3</td>
<td>0%</td>
<td>+</td>
<td>9.5</td>
</tr>
<tr>
<td>686</td>
<td>+, CA</td>
<td>3</td>
<td>0%</td>
<td>+</td>
<td>9.5</td>
</tr>
<tr>
<td>687</td>
<td>+, CA</td>
<td>3</td>
<td>0%</td>
<td>+</td>
<td>9.5</td>
</tr>
<tr>
<td>688</td>
<td>+, CA</td>
<td>3</td>
<td>0%</td>
<td>+</td>
<td>6.5</td>
</tr>
<tr>
<td>708</td>
<td>+, CA</td>
<td>5</td>
<td>29%</td>
<td>+</td>
<td>8</td>
</tr>
<tr>
<td>3089</td>
<td>+, CA</td>
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<td>58%</td>
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<td>3099</td>
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<td>+</td>
<td>8</td>
</tr>
<tr>
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<td>5</td>
<td>49%</td>
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<td>8</td>
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<td>41%</td>
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<tr>
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<tr>
<td>759</td>
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<td>-</td>
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</tr>
<tr>
<td>39</td>
<td>+, CF</td>
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<td>0%</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>40</td>
<td>+, CF</td>
<td>2</td>
<td>0%</td>
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<td>715</td>
<td>+, CF</td>
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<td>75%</td>
<td>+</td>
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<td>+, CF</td>
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<td>46%</td>
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<td>1</td>
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<tr>
<td>756</td>
<td>+, CF</td>
<td>4</td>
<td>36%</td>
<td>+</td>
<td>1</td>
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<td>+, CF</td>
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<td>48%</td>
<td>+</td>
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<tr>
<td>781</td>
<td>+, CF</td>
<td>5</td>
<td>35%</td>
<td>+</td>
<td>1</td>
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Key: CA=cell-associated virus, M=mock-infected cells, U=uninfected, CF=cell-free virus, NA=not applicable
Table 2. Detection of viral DNA in blood and lymphoid organs one week post cell-free infection

<table>
<thead>
<tr>
<th>Mouse #</th>
<th>Mouse group</th>
<th>Blood(PF,BC)</th>
<th>BM</th>
<th>Thy</th>
<th>Spl</th>
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<tr>
<td>37-40</td>
<td>NH, I</td>
<td>-</td>
<td>-</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>711</td>
<td>H, I</td>
<td>BC=14,000</td>
<td>110,000</td>
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<td>-</td>
</tr>
<tr>
<td>715</td>
<td>H, I</td>
<td>PF=13,000</td>
<td>3,600</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>755</td>
<td>H, I</td>
<td>-</td>
<td>42,000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>756</td>
<td>H, I</td>
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<td>6,200</td>
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</tr>
<tr>
<td>778</td>
<td>H, I</td>
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<td>1,400</td>
<td>NT</td>
<td>-</td>
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<tr>
<td>781</td>
<td>H, I</td>
<td>-</td>
<td>650</td>
<td>NT</td>
<td>-</td>
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</table>

Key: NH=non-humanized, H=humanized, I=infected, PF=plasma fraction, BC=blood cell fraction, BM=bone marrow, Thy=thymus, Spl=spleen, (-)=below limit of detection, NT=not tested. Plasma and blood cell fractions reported in DNA copies/ml; BM reported in DNA copies per femur; Thy reported in DNA copies per half thymus; Spl reported in DNA copies per one third spleen. For blood, fractions analyzed are indicated in column headings.
Table 3. Detection of viral DNA in blood after cell-associated infection

<table>
<thead>
<tr>
<th>Mouse #</th>
<th>Mouse group</th>
<th>1 week(PF)</th>
<th>2 weeks(WB)</th>
<th>3 weeks(PF)</th>
<th>4 weeks(PF;BC)</th>
<th>5 weeks(PF)</th>
<th>6 weeks(PF;BC)</th>
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<td>NH, I</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>685-688</td>
<td>NH, I</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>698, 3095, 3096, 3098</td>
<td>H, M</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>708</td>
<td>H, I</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3089</td>
<td>H, I</td>
<td>3,200</td>
<td>5,200</td>
<td>68,000</td>
<td>-</td>
<td>-</td>
<td>1,900; -</td>
</tr>
<tr>
<td>3090</td>
<td>H, I</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-; 46,000</td>
</tr>
<tr>
<td>3092</td>
<td>H, I</td>
<td>-</td>
<td>-</td>
<td>33,000</td>
<td>130,000; 290,000</td>
<td>2,100</td>
<td>-; 77,000</td>
</tr>
<tr>
<td>3099</td>
<td>H, I</td>
<td>-</td>
<td>-</td>
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<td>15,000; -</td>
<td>1,200</td>
<td>-</td>
</tr>
<tr>
<td>3100</td>
<td>H, I</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1,200</td>
<td>-; 9,700</td>
</tr>
</tbody>
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Key: NH=non-humanized, H=humanized, I=infected, M=mock-infected, PF=plasma fraction, BC=blood cell fraction, WB=whole blood, (-)=below limit of detection. Plasma and blood cell fractions reported in DNA copies/ml. Not all samples were analyzed by Q-PCR each week. Blood fractions analyzed are indicated in column headings.
Table 4. Detection of viral DNA in lymphoid organs from mice sacrificed 6 ½ to 9 ½ weeks post cell-associated infection

<table>
<thead>
<tr>
<th>Mouse #</th>
<th>Mouse group</th>
<th>Bone Marrow</th>
<th>Thymus</th>
<th>Lymph Node</th>
<th>Spleen</th>
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<td>-</td>
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<tr>
<td>685-688</td>
<td>NH, I</td>
<td>-</td>
<td>NT</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>698</td>
<td>H, M</td>
<td>-</td>
<td>NT</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>3095</td>
<td>H, M</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3096</td>
<td>H, M</td>
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<tr>
<td>3098</td>
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<td>3090</td>
<td>H, I</td>
<td>-</td>
<td>NT</td>
<td>NT</td>
<td>-</td>
</tr>
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<td>H, I</td>
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<td>8,100</td>
<td>2,800</td>
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<td>3100</td>
<td>H, I</td>
<td>2,700</td>
<td>NT</td>
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</tbody>
</table>

Key: NH=non-humanized, H=humanized, I=infected, M=mock-infected, (-)=below limit of detection, NT=not tested. Bone marrow reported in DNA copies per femur; Thymus reported in DNA copies per half thymus; Lymph node reported in DNA copies per node; Spleen reported in DNA copies per one third spleen.
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Figure 2. HHV-6A infected carrier cells used in cell-associated transmission to humanized mice. (A-D)
Flow cytometry of (A,C) uninfected and (B,D) HHV-6A infected cells prior to injection into RAG-hu mice.
(A,B) Detection of GFP expression. (C,D) Analysis of CD3 and CD4 expression.
Figure 4. Detection of CD4$^+$CD8$^+$ T cells in blood of HHV-6A infected mice. 
(A-C) Levels of CD4$^+$CD8$^+$ T cells in blood were quantified by flow cytometry. 
(A) uninfected mouse, (B) HHV-6A infected mouse 715 (cell-free transmission, 
and highest incidence of CD4$^+$CD8$^+$ cells). All samples were first gated on 
CD3. (C) Mean levels of CD4$^+$CD8$^+$ cells were quantified in cell-free infected 
mice (n=6), uninfected mice (n=4). Standard error indicated; student’s t test 
used for statistical analysis. *, p ≤ 0.05.