Humanized-BLT mouse model of Kaposi's sarcoma-associated herpesvirus infection

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Lack of an effective small-animal model to study the Kaposi's sarcoma-associated herpesvirus (KSHV) infection in vivo has hampered studies on the pathogenesis and transmission of KSHV. The objective of our study was to determine whether the humanized BLT (bone marrow, liver, and thymus) mouse (hu-BLT) model generated from NOD/SCID/IL2 γ mice can be a useful model for studying KSHV infection. We have tested KSHV infection of hu-BLT mice via various routes of infection, including oral and intravaginal routes, to mimic natural routes of transmission, with recombinant KSHV over a 1- or 3-mo period. Infection was determined by measuring viral DNA, latent and lytic viral transcripts and antigens in various tissues by PCR, in situ hybridization, and immunohistochemical staining. KSHV DNA, as well as both latent and lytic viral transcripts and proteins, were detected in various tissues, via various routes of infection. Using double-labeled immune-fluorescence confocal microscopy, we found that KSHV can establish infection in human B cells and macrophages. Our results demonstrate that KSHV can establish a robust infection in the hu-BLT mice, via different routes of infection, including the oral mucosa which is the most common natural route of infection. This hu-BLT mouse not only will be a useful model for studying the pathogenesis of KSHV in vivo but can potentially be used to study the routes and spread of viral infection in the infected host.

humanized mice | HHV-8 | mucosa transmission

The Kaposi's sarcoma (KS)-associated herpesvirus (KSHV), also known as the human herpesvirus 8, was first identified from KS tissues in 1994 (1). It is the etiologic agent for KS and is also associated with primary effusion lymphoma (PEL) and multicentric Castlemann's disease (2). More recently it was also found to be associated with KSHV-associated inflammatory cytokine syndrome (3). Although substantial progress has been made in characterizing the virus, there are still many unanswered questions such as how KSHV infection can lead to disease manifestation and whether latent or lytic induction of KSHV are associated with malignancies. One of the reasons is a lack of a good small-animal model to study KSHV infection in vivo, which has hampered studies on how KSHV infects, spreads, and how it interacts with the host and ultimately leads to disease pathogenesis. Moreover, currently there is no vaccine against KSHV infection, and there is need for an effective animal model to evaluate the efficacy of vaccines if they are developed and for the testing of antiviral regimens.

An ideal model should have relatively short generation time, reproduce rapidly, be inexpensive to maintain and house, and be easy to manipulate. An example is a rodent model that can be infected by KSHV effectively. Several small-rodent models have been tested for KSHV infection. The models include transplantation with both human KSHV-infected B lymphoma cells and primary human peripheral blood mononuclear cells in the SCID mouse (4), injection of KSHV into the human skin engrafted or the transplant of the SCID mice (5, 6), or injection of KSHV or KSHV-infected cells into the nonobese diabetic (NOD)/SCID mice (7, 8). However, a better understanding of KSHV transmission, early events of viral infection,

its pathogenesis, its interaction with the host, and the development of disease requires an in vivo model that supports natural routes of viral infection, a long-term sustainable infection involving both latent and lytic viral gene expression, and the infection of target tissues and cells that reflect those of human infection. The mouse models used so far have not been able to achieve such goals.

Recently, a new generation of humanized mouse, the BLT (bone marrow, liver, and thymus) mouse (hu-BLT) generated from NOD/SCID/IL2 γ (NSG) mouse, has been shown to be an excellent model for studying human viral infections (9). This model has been shown to harbor a sustained high level of total human immune cells and is also the only model that can generate the human mucosal immune systems. In this study we found that BLT mice can be infected by rKSHV.219 via intraperitoneal, oral, and vaginal routes of inoculation. KSHV DNA, latent protein LANA, and lytic protein K8.1 were readily detectable in various tissues of the infected hu-BLT mice over 1- or 3-mo periods after infection. We found that the virus can establish both lytic and latent infections in human B cells and macrophages in the spleens, whereas infected cells in the skin were predominantly latently infected macrophages. These results demonstrate that KSHV can establish both lytic and latent infections efficiently in the hu-BLT mouse model and will be useful for studying the pathogenesis and transmission of KSHV in vivo.

Results

Infection of the hu-BLT Mice by KSHV. Before the inoculation by KSHV the peripheral blood cells of the studied animals were tested periodically by flow cytometry to monitor for the presence of human leukocytes (hCD45⁺), mouse leukocytes (mCD45⁺), human T cells (hCD45⁺ hCD3⁺), and human B cells (hCD45⁺

Significance

The Kaposi's sarcoma-associated herpesvirus (KSHV) is the etiologic agent of Kaposi's sarcoma, which is the most common malignancy found in AIDS patients. The lack of a good animal model to study KSHV infection in vivo has hampered studies on the pathogenesis and transmission of KSHV. We report here that a humanized BLT (bone marrow, liver, and thymus) mouse model can support a robust KSHV latent and lytic infection, via transmission routes that occur during natural infection in humans. This humanized BLT mouse model will be useful for studying the pathogenesis and transmission of KSHV in vivo.

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hCD19⁺) at 2- or 4-wk intervals after reconstitution. The mice used for inoculation by KSHV were found to have an average of approximately 80% human CD45⁺ leukocytes among the total leukocytes in peripheral blood cells at 12 wk after reconstitution (Table 1). An example of one of the killed animals is shown in Fig. 1A. Human MHC-1⁺ and human CD45⁺ cells were also readily detectable in other tissues, such as spleens and skins. On the average there were 50.7% $\pm$ 23.8% human CD45⁺ cells and 19.7% $\pm$ 7.8% mouse CD45⁺ in the spleens with six orally infected animals analyzed as determined by flow cytometry. An example of the flow data of the spleen of a typical BLT mouse is shown in Fig. 1B, and an example of immunofluorescence staining in the skin tissue is shown in Fig. 1C.

To determine whether hu-BLT mice can be infected by KSHV, mice were divided into three groups, with each inoculated with 2.5×10^5 infectious units of KSHV via three routes of infection as shown in Table 1. Half of the i.p. and vaginal, and six of nine orally infected animals were inoculated twice at 0 and 2 wk; the rest of the animals were inoculated four times at 0, 2, 6, and 10 wk. The animals were killed to test for KSHV infection at 2 wk after the final inoculation. All animals inoculated with either two or four doses of KSHV were found to be infected via either routes of inoculation. KSHV DNA was found in various tissues tested, such as lungs, spleens, lymph nodes, and skins (Table 1). No viral DNA was detected in control mock-infected animals.

The Human Cells in the Spleens of hu-BLT Mice Inoculated Orally by KSHV Can Be Infected. Because the saliva and oral mucosal infections have been shown to be the most likely routes of KSHV transmission in humans, and B cells were shown to be a target of infection, we first determined the effectiveness of infection of spleen cells in animals inoculated orally by flow cytometry. Fig. 2A summarizes the levels of infection of six orally infected animals at 4 wk after infection: an average of 3.72% $\pm$ 1.65% of the total spleen cells and 7.12% $\pm$ 0.88% of the total human cells were infected, compared with only 0.29% $\pm$ 0.07% of the total mouse cells in the spleens. An example of the flow cytometry data of the spleen of one of the infected animals is shown in Fig. 2B. For this animal 5.27% of total spleen cells was found to be infected by KSHV.219 and expressed GFP encoded by the virus (Fig. 2B). Most infected cells were found to be human cells; among them 6.61% were infected human cells, and 0.21% were infected mouse cells.

To confirm that the cells infected by KSHV were expressing viral transcripts, in situ hybridization (ISH) was carried out using labeled viral RNA probes. The LANA antisense RNA probe was used to detect viral LANA mRNA, and a mixture of two antisense RNA probes of viral gB and K8.1 lytic genes were used to detect lytic genes expression. Both latent and lytic gene expressions were detected in spleens of mice that were inoculated via various routes and doses of KSHV. Very clear hybridization signals using the LANA-specific probe were detected in spleen tissue, and an example of an orally infected animal is shown in Fig. 3 and Fig. S1, with LANA-specific (Fig. 3A, a) or with gB- and K8.1-specific probes (Fig. 3A, c) at 12 wk after infection. Expression of both latent and lytic genes was found to be at comparable levels. No signal was detected using either LANA sense probe (Fig. 3A, b) or gB and K8.1 sense probes (Fig. 3A, d) as

Fig. 1. Levels of human immune reconstitution in hu-BLT mice at 12 wk after surgery. (A) PBLs were incubated with antibodies and then analyzed by flow cytometry. Approximately 2×10^4 total live cells were evaluated for the coexpression of either hCD45, mCD45, hCD3, or hCD19. (B) Splenic single-cell suspensions were analyzed by multicolor flow cytometry. Approximately 5×10^4 total live cells were evaluated for the coexpression of either hCD45, mCD45, hCD3, or hCD19. (C) Skin tissues were analyzed by immunofluorescence using rabbit antibodies against hMHC-I (red) or hCD45 (red). DAPI (blue) was used as counterstain. (Scale bars, 20 μ m.)

controls. These results imply that the infected spleen cells expressed both latent and lytic genes.

The infected spleen cells were also found to be expressing viral latent and lytic proteins by immunohistochemical staining (IHCS), using antibodies specific for LANA and K8.1 proteins (Fig. 3B). The anti-LANA antibody detected a typical punctate staining of LANA in the nuclei of infected cells (Fig. 3B, a), with a mean of approximately 23.4 cells/mm² LANA-positive cells in infected spleen tissues, and no staining was seen in the isotype control or uninfected spleen tissues (Fig. 3B, b and c). Similarly, anti-K8.1 antibody showed a consistent membrane and cytoplasmic staining in the lytically infected spleen cells (Fig. 3B, d; mean, 78.4 cells/mm²), and no staining was found in uninfected spleen tissues (Fig. 3B, e) or with isotype control (Fig. 3B, f). In contrast to LANA, substantially more lytically infected cells expressing K8.1 antigen were detected. This suggests there are more lytically infected cells than latently infected cells in the spleens of the infected mice.

The Human Cells in the Skins of hu-BLT Mice Inoculated Orally by KSHV Can Be Infected by KSHV. Endothelial cell is a primary target for KSHV and is involved in KS development. Because KSHV DNA was found in the skin by PCR, we then tested the skins of infected animals using both ISH and IHCS. An example of the skin from an orally infected animal is shown in Fig. 4A and Fig. S2. In contrast to the infected spleen, the gB- and K8.1-specific probes revealed relatively few positive cells in the skin tissues (Fig. 4A, c), and the LANA-specific probe detected many more cells expressing a strong signal (Fig. 4A, a) than the lytic gene probe. This result suggests that the majority of the KSHV-infected cells in the skins were in the latent phase of infection.

To confirm the expression of viral latent and lytic proteins in the skin tissues, IHCS using anti-LANA and anti-K8.1 was carried out. As expected, LANA- and K8.1-expressing cells were

Table 1. Reconstitution and infection of the hu-BLT mice by KSHV

Inoculation route	n	Inoculation times (1 or 3 mo)	% hCD45 ⁺ in total leukocytes	% T in hu-leukocytes	% B in hu-leukocytes	PCR positive (<i>orf26</i>)
Oral	9	2–4 times	80.8 (65.3–89.1)	39.5	59.8	Skin, spleen, lung
i.p.	4	2–4 times	80.5 (77.6–81.1)	58.5	39.0	Skin, spleen, liver, kidney, lung, ileum, jejunum
Vaginal	4	2–4 times	80.2 (70.2–88.7)	70.5	26.1	Skin, lymph nodes, ileum
Control (PBS)	5	2 times	81.1 (76.5–86.4)	42.0	55.2	—

A

Cells	% of total cells mean $\pm$ SD	% of human cells mean $\pm$ SD	% of mouse cells mean $\pm$ SD
GFP ⁺ (KSHV)	3.72 $\pm$ 1.65	7.12 $\pm$ 0.88	0.29 $\pm$ 0.07

Fig. 2. Determination of KSHV infection of the spleens of hu-BLT mice infected by KSHV orally at 4 wk after infection by flow cytometry. (A) Determination of KSHV infection efficiencies of the spleen tissues at 4 wk after infection ($n = 6$). (B) Splenic single-cell suspensions were measured by flow cytometry. Approximately 3×10^4 total live cells were evaluated for the coexpression of GFP and hMHC-I.

detected in the skin tissues of the animals regardless of the route and dosage of infection. Again, very few infected skin cells were expressing K8.1 lytic antigen. An example of the skin tissue of an orally infected mouse is shown in Fig. 4*B* and Fig. S2. A majority of the skin cells from KSHV-infected cells showed LANA staining (Fig. 4*B*, *a*), with a mean of 38.1 cells/mm² LANA-positive cells in infected skin tissues. In contrast, very few cells were found to be expressing K8.1 (Fig. 4*B*, *d*; mean, 2.8 cells/mm²). Additionally, no positive cell was found in the isotype controls (Fig. 4*B*, *b* and *e*) or with control uninfected skin tissues (Fig. 4*B*, *c* and *f*). Our results suggest that in contrast to the infected spleen cells, most infected cells are in the latent phase of infection.

Identification of KSHV-Infected Cell Types in the Spleens of Orally Infected hu-BLT Mice by Multicolor Flow Cytometry and by Double-Label Immunofluorescence Analysis. To identify the specific human cell types that are infected by KSHV in the BLT mice, we first analyzed the infected cells in the spleens by flow cytometry. Our flow analyses showed that an average of $3.41\% \pm 1.67\%$ of total spleen cells of the six orally infected animals analyzed were infected human leukocytes (hCD45⁺), and $3.07\% \pm 1.68\%$ of all analyzed spleen cells were infected human B cells (hCD45⁺ hCD19⁺) (Fig. 5A). No CD3⁺ T cells were found to be infected. This suggests that most of the infected splenic leukocytes were in fact human B cells. This was confirmed by analyzing the KSHV-infected MHC-positive human cells. An average of $95.8\% \pm 1.4\%$ of the infected human cells were human leukocytes, and an average of $83.9\% \pm 6.8\%$ were B cells (Fig. 5A). In addition, an average of $39.9\% \pm 15.9\%$ infected human cells were found to be human CD31⁺ cells (Fig. 5A). This suggests that certain subpopulations of the leukocytes can also express CD31, an endothelial cell marker that has been reported to be expressed by macrophages/monocytes and some subpopulation of lymphocytes (10). Because infected human T cells were undetectable, thus the infected CD31⁺ cells could be macrophages/monocytes, some subpopulation of B cells, and/or endothelial cells. An

example of the spleen of an orally infected animal is shown in Fig. 5B. Through our flow analysis we concluded that the major population of the infected human cells in spleens of the infected BLT mice is B cells.

To confirm our flow analyses results and to identify the specific cell types, we analyzed the spleen tissues of animals infected orally by confocal microscopy, using double-label immunofluorescence (IFA) with anti-LANA or anti-K8.1 antibodies in combination with antibodies against different human cell-surface markers. As expected, LANA- and K8.1-expressing cells were detected in the splenic tissues. We found that hMHC-1⁺, hCD45⁺ (leukocytes), hCD20⁺ (B cells), hCD68⁺ (macrophages), and hCD31⁺ (endothelial cells) were expressing LANA (Fig. 6A and Fig. S3). In addition these same cell types were also found to be K8.1 positive (Fig. 6B and Fig. S3). These results suggest that the different human cell types found in the spleens can undergo both latent and lytic infection. As for hCD31⁺ KSHV-infected cells, they do not resemble endothelial cells morphologically (Fig. 6A and B) but appear to be macrophage-like. To confirm that endothelial cells can also be infected, we have tested an additional endothelial cell marker, VEGFR2. However, results similar to those seen with CD31 staining were obtained (Fig. S4). Thus, at this point it is not clear whether there are substantial levels of human endothelial cells present in the spleen tissues and whether they could be infected by KSHV. Our results nevertheless suggest that KSHV can establish both lytic and latent infection in both human B cells and macrophages in the spleens of hu-BLT mice infected orally. Infection of CD3⁺ human T cells was undetectable.

Identification of KSHV-Infected Cell Types in the Skin Tissues of Orally Infected hu-BLT Mice by Double-Label IFA Analysis. Double-label IFA was also used to identify KSHV-infected cell types in skin tissues of the orally infected animals. We found that human MHC-1⁺, CD45⁺, CD68⁺, and CD31⁺ cells in the skin tissues were also positive for LANA (Fig. 7 and Fig. S5). Interestingly, in contrast to the spleen, infected human CD20⁺ B cells were not found. Also in contrast to the spleen, majority of infected cells were LANA-positive, and there were very few lytically infected K8.1-expressing cells in the skin tissues analyzed, supporting our immunohistochemistry analysis demonstrating that most of the infected cells in the skin were expressing LANA antigen only (Fig. 4B). Similar to the results with the spleen tissues, we cannot conclude that the endothelial cells were present and infected in the skin even though they expressed CD31 (Fig. 7). In summary, the KSHV-infected skin cells seemed to be predominantly latently infected human macrophages.

Discussion

Currently the hu-BLT mouse model has been used mostly to study HIV-1 and a limited number of other viruses (9, 11–14). Little is known whether this model can be used to study KSHV infection, for which there is no good small-animal model to study its infection and disease pathogenesis. We demonstrated in this study that the hu-BLT mice can be infected via several known

Fig. 3. Expression of KSHV latent and lytic genes and proteins in the spleens of hu-BLT mice infected orally. (A) KSHV latent and lytic gene expression in the spleen of a hu-BLT mouse by ISH at 12 wk after infection. Probe for latent gene LANA was exposed for 8 d (*a*, LANA anti-sense probe; *b*, LANA sense probe), and 16 d for the lytic gene probes (*c*, K8.1 and gB antisense probes; *d*, K8.1 and gB sense probes). (B) KSHV latent and lytic proteins expression in the spleens at 4 wk after infection. The spleens were from infected (*a*, *b*, *d* and *e*) or from PBS control mice (*c* and *f*). IgG (*b* and *e*). (Scale bars, 100 μ m in larger panels).

Fig. 4. KSHV latent and lytic genes and proteins expression in the skins of hu-BLT mice inoculated orally by KSHV at 12 wk after infection. (A) KSHV latent and lytic gene expression in the skin of a hu-BLT mouse by ISH. Thin sections of the spleen were hybridized with ³⁵S-labeled riboprobe specific for KSHV LANA gene after 8 d exposure (*a*, LANA antisense probe; *b*, LANA sense probe), and 16 d exposure for lytic genes (*c*, K8.1 and gB antisense probes; *d*, K8.1 and gB sense probes). (B) Detection of KSHV latent and lytic proteins expression by IHCS in the skins. The skins were collected from the infected (*a*, *b*, *d*, and *e*) or control PBS mice (*c* and *f*). Skin sections were indicated LANA⁺ cells. (Scale bars, 100 µm in large)

natural routes of infection, such as via oral and vaginal mucosal routes. We also found that a large number of human B cells and macrophages, which are known natural human host cells, were infected. Our finding represents an important step forward toward developing a robust small-animal model for KSHV infection and disease pathogenesis.

It has been shown that KSHV DNA could be detected in rhesus macaques inoculated with KSHV-infected PEL cells, but it replicated to very low levels, and viral mRNA was not detectable (15). In addition, Chang et al. (16) recently reported that KSHV can establish persistent infection in marmosets and could potentially serve as an animal model. However, given the difficulty in working with nonhuman primates there is still a great need to develop small-animal models, such as a rodent model, that can be used to study KSHV infection and pathogenesis. There have been several earlier attempts to use humanized mouse models to study KSHV infection: the hu-PBL with injected human peripheral blood lymphocytes (PBLs) (4), the hu-HSCs mice engrafted with hematopoietic stem cells (HSCs) (7), and the SCID-hu mice implanted with human liver and

thymus (6). However, these models have low levels and limited functionality of the human immune cells, and the extent of infection and gene expression was limited because of the limited reconstituted human cell repertoire. The new generation of BLT model uses the NSG parental mouse strain, which yielded more severely immunocompromised mice to achieve far superior human cell engraftment (11). This is also the only mouse model that can generate the human mucosal immune system and a human MHC restricted antigen-specific humoral and cellular response (17).

In this study we found that animals inoculated with only two doses of KSHV can be infected systemically by either oral or vaginal routes of infection. The infected cells were found to be mainly of human origins, but a few KSHV-infected cells were found to be negative for human MHC class I in the spleen. This observation of infected murine cells is consistent with a previous study, which has shown that KSHV can infect the mouse cells but that the infected cell numbers were low (8). However, it is unlikely that mouse cells can support a sustainable KSHV infection because it was shown by Austgen et al. (18) that there are multiple postentry blocks to KSHV lytic replication in murine cells, and infected murine cells were associated with apoptotic cell death.

Our study here with the BLT mice showed that both latent and lytic infection can be established in the infected animals regardless of the route of infection. Interestingly, different levels of lytic and latent infections can be established in different tissues. Both lytic and latent infection can be observed in the spleen. In contrast, in the skins most infected cells were latent and expressed LANA. This difference in latent vs. lytic replication could reflect the tissue and cell type specificity, such that in the spleen latent infection occurs in the spindle cells but lytic replications are associating with B cells. However, in the skin tissues, the positive cells are mainly macrophages. At this point it is not clear whether the CD31⁺ and VEGFR2⁺ human cells are in fact endothelial cells because even though they were stained positive by endothelial cell markers they resemble macrophages morphologically. It is possible that some macrophages could be expressing endothelial cell markers in our infected animals. It has been reported that transplanted human embryonic stem cell-derived CD34⁺ cells can develop into human endothelial cells in the liver of BLT mice (19); whether the human endothelial cells are present in the skins and spleens of the BLT mice and whether they can be infected by KSHV will need further investigation.

Despite the robust infection of the hu-BLT mice, we were not able to detect any humoral immune response against KSHV when tested up to 3 mo after infection. This is not unexpected because HIV-specific antibodies were delayed in the hu-BLT model and can only be detected after 12 wk of infection (20). Because the natural history of primary KSHV infection remains unknown, and the humoral response against KSHV is known to be nonrobust and transient (21), the KSHV serological responses in this model need to be investigated further over a longer period

Fig. 5. Identification of KSHV-infected cell types in the spleens of hu-BLT mice at 4 wk after oral infection by flow cytometry. (A) KSHV infection efficiencies and infected cell populations in the spleen tissue at 4 wk after infection ($n = 6$). (B) Splenic single-cell suspensions were measured. The splenic single-cell suspensions were incubated with hCD45, hCD19, and hCD31 mouse monoclonal antibodies and rabbit anti-GFP antibody. Approximately 3×10^4 total live cells were evaluated using a combination of two antibodies.

Fig. 6. Identification of KSHV-infected cell types in the spleens of hu-BLT mice inoculated orally using double-label IFA. (A) Identification of the cell types infected by KSHV latently (LANA⁺) in the spleen tissues. (B) Identification of the cell types infected by KSHV lytically (K8.1⁺) in the spleen tissues. Double-labeled IFA was performed using mouse antibodies to LANA (green) at 1:100 dilution or to K8.1 at 1:1,000 dilution (green), and rabbit monoclonal or polyclonal antibodies to human MHC-I, CD45, CD20, CD68, or CD31 (red). DAPI (blue) was used as counterstain. (Scale bars, 10 μ m.)

of infection. In addition, none of our infected animals have developed any KS-like lesions, lymphomas, or any other specific symptoms known to be related to KSHV infection. Although the present study focused on establishing an in vivo system that supports KSHV infection and the recapitulation of both latent and lytic gene expression, whether this model can be used to examine viral-related pathology remains to be established.

In conclusion, we report here that a humanized BLT mouse model generated from NSG mice can support robust KSHV latent and lytic infection, via transmission routes that occur during natural infection in humans. Our data showed latent and lytic viral transcripts, and viral protein expressions were detected in various tissues, including spleen and skin tissues over a 1- or 3-mo time course. Interestingly, mice were found to be infected via several routes of infection tested, including via the oral mucosal route. Furthermore, we found that KSHV can establish infection

in human B cells and macrophages in this model. This hu-BLT mouse will be a useful model not only for studying the pathogenesis of KSHV in vivo but can potentially be used to study the routes and spread of the virus in the infected host.

Materials and Methods

Generation of hu-BLT Mice. Hu-BLT mice were generated by following the previously published protocol (22). Six- to eight-week-old NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NOD/SCID/IL2r γ null, NSG) mice (The Jackson Laboratory) were purchased and maintained in pathogen-free conditions at University of Nebraska-Lincoln Life Sciences Annex. Human fetal livers and thymus tissues were procured from Advanced Bioscience Resources. On the day of surgery, mice received 12 cGy/g of mouse body weight with an RS200 X-ray irradiator (Rad Source Technologies). The mice were transplanted with two pieces of human fetal liver and one piece of thymic tissue fragments under the left kidney capsules, followed by injection of $1.5\text{--}2.3 \times 10^5$ fetal liver-derived CD34⁺ HSCs i.v. It took approximately 12–16 wk before the mice were ready

Fig. 7. Identification of the cell types infected by KSHV latently in the skins of hu-BLT mice inoculated orally using double-label IFA. Double-labeled IFA was performed using mouse antibody to LANA (green) at 1:500 dilution, and rabbit monoclonal or polyclonal antibodies to human MHC-I, CD45, CD68, or CD31 (red). DAPI (blue) was used as counterstain. (Scale bars, 10 μ m.)

to be challenged with KSHV, when the human leukocytes ratio to total leukocytes was more than 50% in peripheral blood.

Viral Inoculations. Recombinant KSHV expressing GFP (rKSHV.219) (kindly provided by Dr. J. Vieira, University of Washington, Seattle, WA) was used to inoculate hu-BLT mice (23). A total of 2.5×10^5 infectious units of rKSHV.219 were used for each mouse; the animals were inoculated either i.p. ($n = 4$), orally ($n = 9$), or through an intravaginal route ($n = 4$). In addition, five control mice were inoculated with PBS either i.p. ($n = 2$), orally ($n = 2$), or through an intravaginal route ($n = 1$). Half of the i.p. and i.v., and six of nine oral-group mice, except the control, were inoculated twice at 0 and 2 wk. The remaining animals were inoculated four times at 0, 2, 6, and 10 wk. All studied animals were killed to test for KSHV infection at 2 wk after the final inoculation.

Peripheral Blood and Tissue Analyses. PBLs were obtained before inoculation and at various time points for flow cytometry analyses. Killed mice tissues were dissected, fixed in SafeFix II (Fisher HealthCare) or 4% (vol/vol) paraformaldehyde and then embedded in paraffin for analyses. Fresh tissues were frozen and stored at -80°C for DNA isolation. Fresh spleen tissues were collected for subsequent flow analyses.

DNA Extraction and PCR Amplification from hu-BLT Mice Tissue for KSHV Detection. DNA was extracted from mice tissue samples using the PureGene Genomic DNA Purification Kit (Qiagen) according to the manufacturer's protocol. DNA was analyzed for KSHV DNA by PCR of the *orf26* gene using a previously described nested PCR protocol (24).

Immunohistochemical and Immunofluorescent Stainings. The IHCS used has been described in detail previously (25). Briefly, slides were incubated in 3% hydrogen peroxide for 30 min to block endogenous peroxidase activity if HRP-conjugated antibody was used, followed by incubating in mouse IgG blocking reagent (AffiniPure Fab Fragment Goat anti-mouse IgG; Jackson ImmunoResearch Laboratories) for 2 h, then incubated for overnight at 4°C with mouse anti-LANA (1:1,000 dilution, kindly provided by Dr. Bala Chandran, Rosalind Franklin University of Medicine and Science, Waukegan, IL) or with mouse anti-K8.1 (1:1,000 dilution; 2A3, Advanced Biotechnologies) in blocking reagent. After incubation with anti-mouse polymer-HRP-labeled secondary antibody (Dako) or with anti-mouse MACH2 universal polymer-alkaline phosphatase-labeled secondary antibody (Biocare Medical). The slides were then incubated in DAB or Vulcan Fast Red (Biocare Medical) for color development. The complete list of antibodies used can be found in *SI Materials and Methods*. Confocal microscopy was used to identify the infected cell types.

Quantification of KSHV-Infected Cells by Immunohistochemistry. The frequency of KSHV latently (LANA⁺) or lytically (K8.1⁺) infected cells was quantified using a positive pixel count algorithm in Aperio's Spectrum Plus analysis program (version 9.1; Aperio ePathology Solutions) as described previously (26). Briefly, immunohistochemically stained tissue sections were digitized using Scanscope. The LANA- or K8.1-positive cells in digital slides were quantified using a positive pixel count algorithm. The parameters of the algorithm were tuned to match the markup image of LANA- or K8.1-positive staining accurately over stain. Once the parameters were set, the algorithm was applied automatically to all digital slides to measure the number of LANA- or K8.1-positive cells (cells⁺/mm²). The mean values of LANA- or K8.1-positive cells in the orally infected tissues ($n = 9$) were calculated.

Riboprobe Preparation and ISH. KSHV-specific probes were generated by amplifying ~700-bp LANA, K8.1 and gB DNA fragments using LANA expression plasmid pSG-flag-LANA (kindly provided by Dr. Kenneth M. Kaye, Harvard Medical School, Boston, MA), K8.1 expression plasmid pcDNA3.1 (+)-K8.1, and gB expression plasmid pcDNA3.1(-)-gB (initial plasmids both kindly provided by Dr. Bala Chandran, Rosalind Franklin University of Medicine and Science) as templates, respectively. Each fragment was subcloned into a pGEM-T-easy vector (Promega). The resulting clones were then linearized on either side of the insert with restriction enzyme to produce linear templates for runoff transcription of antisense and sense RNAs. Radiolabeled riboprobes were synthesized by incorporating ³⁵S-UTP using the Promega transcription system, and ISH was conducted according to published methods (25, 27).

Multicolor Flow Cytometry. Preparation of a single-cell suspension from spleen using collagenase type IV (Sigma-Aldrich) has been described elsewhere (28). After red blood cells were lysed, all cells were incubated in 80 μL of the blocking reagent with 0.5 $\mu\text{g}/10^6$ cells of mouse Fc γ III/II receptor blocker (BD Biosciences-Pharmingen) for 10 min. Cell aliquots were incubated for 30 min in the dark at 4°C with fluorochrome-conjugated antibodies, at 0.5 μg antibodies per 10^6 cells or according to the manufacturer's instructions. The complete list of antibodies used can be found in *SI Materials and Methods*. Stained cells were analyzed using a BD FACSAria III (BD Biosciences) and FlowJo software (version 7.6.4; Tree Star).

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