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Kaposi's sarcoma herpesvirus-induced endothelial cell reprogramming supports viral persistence and contributes to Kaposi's sarcoma tumorigenesis

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Kaposi's sarcoma (KS) is an endothelial tumor causally linked to Kaposi's sarcoma herpesvirus (KSHV) infection. At early stages of KS, inflammation and aberrant neoangiogenesis are predominant, while at late stages the disease is characterized by the proliferation of KSHV-infected spindle cells (SC). Since KSHV infection modifies the endothelial cell (EC) identity, the origin of SCs remains elusive. Yet, pieces of evidence indicate the lymphatic origin. KSHV-infected ECs display increased proliferative, angiogenic and migratory capacities which account for KS oncogenesis. Here we propose a model in which KSHV reprograms the EC identity, induces DNA damage and establishes a dysregulated gene expression program involving interplay of latent and lytic genes allowing continuous reinfection of ECs attracted to the tumor by the secretion of virus-induced cellular factors.

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Kaposi's sarcoma and identification of its associated herpesvirus

Kaposi's sarcoma (KS) was named after a Hungarian dermatologist, Moritz Kaposi, who first described five cases of this multifocal, pigmented tumor of the skin. KS remained a rare disease until the 1980s when an aggressive form was observed among the AIDS patients. The high number of cases together with the bad prognosis associated with AIDS-KS prompted researchers on studying the disease in detail. Particular efforts were put into the identification of KS causal agent and in 1994 Chang and Moore identified it as a newly discovered virus named Kaposi's sarcoma herpesvirus (KSHV) [1]. Classified as a gamma2 herpesvirus [2], KSHV has been subsequently associated also with two lymphoproliferative disorders: primary effusion lymphoma (PEL) and multicentric castleman disease (MCD) [3,4].

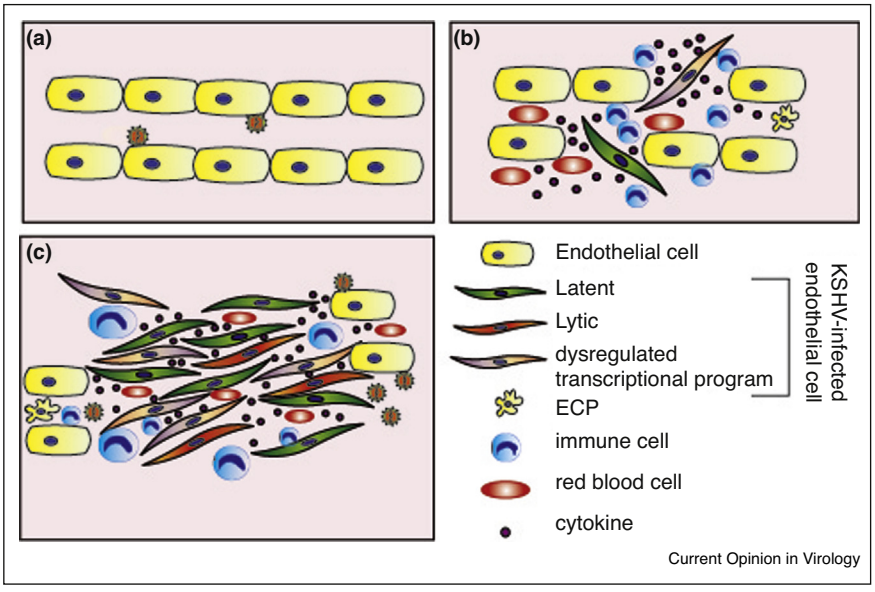
KSHV displays a biphasic life cycle with a latent and a lytic phase. Although latency represents the default mode of persistence in the tumor cells, both phases contribute to KS pathogenesis. Since many reviews [5–8] recently discussed the molecular mechanisms of KS induced tumorigenesis, here we will focus on the reprogramming and manipulation of endothelial cells and its contribution to KS pathogenesis.

KS: an angiogenic, inflammatory tumor of the endothelium

Different forms of KS can be distinguished according to their epidemiological characteristics. In the sub-Saharan Africa KS is a common tumor among men and children (epidemic KS), whereas in the Mediterranean basin a milder form of KS affects primarily elderly men (endemic KS) [9]. The clinical features of KS become more aggressive when it occurs in immunosuppressed patients, such as transplant recipients (iatrogenic KS) and AIDS patients (AIDS KS) [10,11]. In these individuals, the lesions are life threatening because of the visceral involvement [11].

Microscopically, the different forms of KS are indistinguishable and display rather stage-specific features. At the early phase, KS manifests as patches characterized by abundant and aberrant angiogenesis. The abnormal blood vessels are leaky, thus accounting for the extravasation of erythrocytes and inflammatory cells that populate the lesion (Figure 1). The early KS patch appears as a flat, red macula (located in the limbs or face) and no evident tumor mass is present. In these patches, as little as 10% of the tumor is composed of KSHV-infected cells. When progressing toward advanced stages, KS develops into plaques and then into nodules. Here, 90% of the cells are KSHV-positive. The tumor mass is macroscopically evident and composed of so-called spindle cells (SCs) [12] that is, KSHV-infected cells

Figure 1



Schematic model of a KS lesion **(a)** KSHV virions infect endothelial cells. **(b)** Early KS lesion: KSHV infected endothelial cells display the typical spindle morphology and produce pro-angiogenic and inflammatory cytokines that chemotactically attract immune cells to the tumor site. Aberrant angiogenesis takes also place and causes the extravasation and accumulation of red blood cells. **(c)** Late KS lesion: the tumor is composed of KSHV-infected SCs. Majority of the SC are in a latent/dysregulated lytic phase, produce inflammatory and angiogenic chemokines and express many oncogenic viral proteins. Only a small subset of SCs are in a lytic phase and they produce new infectious virions for the maintenance of the population of infected cells.

with characteristic, elongated (spindle) morphology (Figure 1).

On the cellular origin of KS spindle cells

Different studies reported that SCs express a number of endothelial cell (EC) markers, for example, CD31, CD34 and factor VIII but they also harbor, to a lesser extent, lineage specific signatures of smooth muscle cells (α-SMA), monocytes/macrophages, dendritic cells as well as fibroblasts and mesenchymal cells [13,14••,15–18].

The gene expression signature of KS SCs indicates an endothelial origin; however, it is still a matter of debate whether the original precursor belongs to the blood or lymphatic lineage. KSHV infection of lymphatic ECs (LECs) triggers the reprogramming toward a less differentiated, immature blood ECs (BECs) phenotype, making KSHV-LECs more similar to BECs than their uninfected counterpart. KSHV-infected BECs, vice versa, express lower levels of blood vascular markers such as CXCR4 and Neuropilin-1 and show an increase in lymphatic markers like Prox1, VEGFR3, podoplanin and LYVE1 (reviewed in [19]). KSHV infection of ECs manipulates the EC differentiation program driving each cell type away from its original, mature phenotype and pushing it toward an undifferentiated, even mesenchymal-like cell type with mixed identity (reviewed in [20••]). As a result, KSHV-infected ECs display tumorigenic properties such as increased angiogenic, invasive and migratory abilities and, although not fully immortalized [21,22], they exhibit a growth advantage over their uninfected counterparts [14••,23].

Several observations suggest that LECs rather than BECs are the main precursor of SCs. First, the localization of KS lesions in tissues rich in lymphatics (e.g. skin and mucosa) [24–26]. Second, KSHV-LECs exhibit, in culture, the elongated (spindling) morphology, resembling KS-SCs, whereas in BECs the morphology is not significantly altered by KSHV infection. Third, LECs can be more efficiently infected and harbor a higher viral copy number [13]. In Addition, the comparison of the gene expression profile of nodular KS lesion with those of BECs and LECs showed that KS signature resembles, at least at this stage, more LECs than BECs [13]. Moreover, three-dimensional culture of KSHV-LEC as spheroids has been shown to recapitulate many of the features found in the KS lesions, among others the differential expression of endothelial and mesenchymal markers in virus-infected cells and the increased tumorigenic invasiveness in the 3D matrix [14••].

Still, the presence of KSHV-infected BECs in KS lesions cannot be ruled out. KSHV-BECs are found in the early

KS patches, where virus-positive cells are lining the walls of aberrant blood vessels. However, in the advanced lesions, although representing the majority of the tumor mass, KSHV-positive SCs were not found in the blood vessels [27]. One could hypothesize that KSHV infects both BEC and LEC at the early stages of the disease, but then KSHV-LECs, more susceptible hosts for the virus, propagate more efficiently and become the predominant cell type in advanced lesions. In agreement with this hypothesis is a study in which Prox1 expression, a marker of lymphatic differentiation (see below), was investigated in AIDS-oral KS. Here, Prox1, almost absent in the early KS patches, increased significantly in the advanced plaque and nodular stages [28•].

Another possibility is that KS-SCs arise from the infection of circulating EC precursors (ECPs), CD34-positive bone marrow-derived cells that home into angiogenic sites where they differentiate into mature ECs [19]. In support of this hypothesis: first, ECPs isolated from KS patients were found positive for KSHV, retained the virus after several passages and were able to sustain lytic replication [29••]; second, ECPs from KS patients displayed higher angiogenic potential *in vitro* [30•]; third, KSHV-positive, CD34+, adherent SCs were found in the blood mononuclear cell fraction [31]; fourth, the multifocal nature of KS lesions occurring independently in different body areas and preferentially in the surgical scares [25,32] or other sites of previous inflammation and angiogenesis, where the ECPs are recruited.

Molecular mechanisms of KSHV-induced trans-differentiation of endothelial cells

The trans-differentiation induced by KSHV represents an abnormal pathological process that can endow ECs with tumorigenic properties (increased proliferation, invasiveness and angiogenic potential), thus contributing to KS oncogenesis. In order to manipulate the EC-lineage identity, KSHV infection interferes with the expression of factors responsible for the differentiation and maintenance of specific EC types.

The transcription factor prospero homeobox 1 (Prox1) has been identified as the master regulator of the LEC fate [33]. Prox1 is required for the development of the lymphatic vasculature [34,35], and it also maintains the LEC cellular identity [36,37]. Ectopic expression of Prox1 in BECs drives the expression of LEC-specific markers (podoplanin, VEGFR3, LYVE1) and suppresses those accountable for the BEC signature [33,38].

KSHV modulates Prox1 expression both in LECs and BECs with opposing results [13,14••,19,39,40]. In BECs Prox1 is not expressed under normal conditions, however, upon KSHV infection, Prox1 expression is induced and activates at least part of the LEC-differentiation program. In LECs, normally expressing high levels of Prox1, KSHV infection results in the downregulation of this transcription factor. The molecular mechanisms that govern Prox1 levels during KSHV infection have been recently elucidated. In KSHV-BECs Prox1 expression is driven by the virus-induced upregulation of IL3Rα, which controls Prox1 through Stat5. In KSHV-LECs, however, IL3Rα does not affect the already high Prox1 expression that is regulated by the Notch1-Hey1 circuit. The KSHV-driven activation of Notch1, triggered by the viral proteins vFLIP and vGPCR [14••,41], is responsible for the downregulation of Prox1 in infected LECs but does not affect Prox1 levels in KSHV-BECs. Thus, the simultaneous activation of Notch1 and ILR3α signaling by KSHV, which contributes to the differential regulation of Prox1 levels, leads to lymphatic reprogramming of BECs and induces blood vascular differentiation in LECs.

Another transcription factor with a pivotal role in KSHV-induced trans-differentiation of ECs is the musculo-aponeurotic-fibrosarcoma oncogene homolog (MAF). Expressed in LECs but not in BECs, MAF represses the BEC-specific genes, thus contributing to the maintenance of the LEC identity [38,39]. In a study aiming to identify new KSHV miRNA targets in LECs, MAF was among the top three downregulated genes upon ectopic expression of the KSHV miRNA cluster [42]. Further analysis showed that suppression of MAF in KSHV-LECs triggered the upregulation of BEC-specific genes such as CXCR4, FTL1 and CXCL12. Although no direct interaction has been shown between MAF and Prox1 it has been speculated that these transcription factors might form a repressor complex that contributes to the suppression of the BEC markers to maintain the LEC phenotype.

Inflammatory microenvironment, lytic reactivation and the unique, viral transcriptional program in LECs

KSHV lytic reactivation is crucial for the virus-induced tumorigenesis. This is demonstrated by the high viral loads in plasma associated with the worst KS prognosis and the clinical data indicating that inhibitors of lytic replication (i.e. ganciclovir and forscarnet) have a preventive effect on KS recurrence [43–45]. However, given that reactivation occurs in as little as *2%* of the SCs and that upon completion of productive lytic replication phase KSHV-infected cell dies [46], how can an event occurring in such a small and destined to die cell population be important for tumorigenesis? To explain these apparently contradictory facts, a so-called paracrine effect has been hypothesized [47]. During the lytic phase virtually all viral ORFs are expressed. Several ORFs can manipulate the cellular signaling thus inducing the secretion of pro-inflammatory and angiogenic factors and modulating the behavior of SCs in the tumor microenvironment. For instance, ectopic expression of a lytic KSHV protein vGPCR in ECs *in vitro* and *in vivo* increases the expression and secretion of several angiogenic and inflammatory

factor [48]. However, KSHV is known to elicit a systematic shut-off of cellular transcripts during the lytic phase [49••]. Therefore, the viral manipulation of the host secretome upon expression of a single viral protein may not faithfully recapitulate what happens in virus-infected cells during lytic replication. Although some transcripts for cytokines (e.g. IL6, IL1) can escape the shut-off, a dramatic reduction in the induction of pro-inflammatory and angiogenic factors was observed when comparing lytic cells expressing vGPCR versus KSHV-infected cells [50•,51].

Lytic reactivation is also important to maintain a population of infected cells within the tumor. ECs, in fact, inefficiently maintain the latent infection and they lose the episome after a few rounds of cell division both *in vivo* and *in vitro* [52••]. Lytic replication therefore would be needed to constantly produce new virions that can infect the uninfected cells in the affected tissue. This can be seen as an interesting strategy evolved by the virus to survive and successfully establish a life-long latent infection in a cell type (endothelial) that the virus is unable to immortalize. Despite numerous attempts to propagate cultures of KS biopsies, there are no patient-derived endothelial cell lines stably harboring KSHV [53–56], while several B cell lines stably and latently infected with KSHV have been established from PEL patients [57]. These experimental observations support the notion that, in order to survive in certain cell types, KSHV adopts strategies that are different from those developed for infected B cells.

Several reports have suggested that, in some cellular contexts a dysregulated viral transcriptional program exists that is not ascertainable to either of the latent or lytic viral phases [58,59,60••,61]. This dysregulated gene expression program is characterized by sporadic expression of lytic markers without efficient production of virions and it was initially observed upon KSHV infection of murine cells [58,59]. In particular, murine bone marrow-derived endothelial progenitors expressed many lytic markers upon KSHV infection, and could generate KS-like tumors in mice [58]. However, the relevance of these models has been questioned since mice are not permissive hosts for KSHV.

More recently, a similar viral transcriptional program has been described also in KSHV-LECs [60••]. Upon infection, differently from BECs, where only the latent genes were detected, LECs displayed a dysregulated viral gene expression pattern. Surprisingly, the KSHV-LECs expressed a variety of lytic ORFs and continued the host cell proliferation, normally not observed during productive lytic replication cycle. This 'leakage' of lytic transcripts in LECs suggests that this could represent an abortive lytic program not producing new virions and not leading to cell death. Although not specifically addressed in the study, it is tempting to speculate that virus-induced host shut-off is not fully effective in the KSHV-LECs, thus allowing the ongoing cell proliferation and perhaps secretion of inflammatory and angiogenic factors. Furthermore, a small amount of infectious progeny was produced, suggesting that a minority of KSHV-LECs spontaneously underwent full lytic replication cycle. Despite the low viral titers, the pool of spontaneously produced progeny virions might be sufficient to expand the population of infected cells and to compensate for the loss of KSHV episomes in dividing cells.

Contribution of the DNA damage response (DDR) to KSHV pathobiology

The association between KSHV infection and KS, PEL and MCD undoubtedly ascribes KSHV to the oncogenic viruses. However, KSHV infection does not induce cellular immortalization, thus suggesting that virus persistence is required to initiate and support tumorigenesis. Activation and manipulation of DDR, a known hallmark of cancers in general [62] represents also a common theme in viral oncogenesis [63,64]. DDR is activated also in the spindle cells in KS lesions, especially in the early (patch) and, to a lesser extent, late (nodular) stages [65••]. This observed checkpoint activation can at least partially explain the low proliferative index of the spindle cells in the KS tumors.

In vitro, DDR has been shown to be induced both during latency establishment and lytic replication phase. During primary infection of ECs, DDR is mounted as early as 30 min post infection. Interestingly, the DDR marker gamma H2AX is localized to the viral genome right after its delivery to the nucleus. This KSHV-induced DDR appeared to be essential for latent gene expression and episome replication [66]. In addition, we and others have reported that in different KSHV-infection models the DDR is instigated upon lytic reactivation [67–69], thus suggesting that the virus replication induces a genomic stress that might represent a driving force in tumorigenesis or that DDR could provide a favorable host cell environment for efficient virus replication.

Although it is known that DDR, first, can function both as an anti-cancer barrier oras a driving force in tumorigenesis, second, is elicited within KS lesions and third, is activated during KSHV lytic replication, the extent of its contribution to KSHV tumorigenesis remains to be elucidated.

Future perspectives

Evidence coming from epidemiological, clinical and *in vitro* studies supports the lymphatic origin of SCs. Here we suggest that KSHV reprograms EC identity to establish a viral gene expression program that allows production of new virions for continuous reinfection of ECs (or ECPs) to maintain a persistent infection (Figure 1). In

addition, the DDR elicited and usurped by the virus to promote its own persistence (in latency) and replication (in the lytic phase), contributes to KS tumorigenesis.

If occurring within a KS lesion, such a model might explain several features of the disease: first, the occurrence of sporadic lytic markers in KS biopsies (due to the LEC-specific viral gene expression program and/or spontaneous lytic reactivation); second, the presence of an angiogenic and inflammatory microenviroment (induced by the sporadic expression of lytic viral genes); third, the maintenance of a population of KSHV-infected cells within the lesion (due to the low-rate spontaneous production of virions); fourth, the presence of DDR activation and low proliferative index in KS lesions. A recent reportsuggests that such a dysregulated transcriptional program occurs at least in a subset of AIDS-KS patients [70••]. Viral profiling of KS biopsies showed that in some tumors a tight latent program was present, whereas in a fraction of the biopsies the viral transcriptional program included expression of both latent and lytic ORFs.

However, several issues remain unclear. For instance, what is the role of circulating KSHV-positive ECPs, how to conceive the 'leaky' lytic program with the virus host shut-off, and what is the role of KSHV reprogramming in the context of virus persistence and what is the contribution of DDR activation and the consequent genomic stress to KS oncogenesis?

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Conflict of interest

The authors declare no conflict of interest.

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