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CUTTING EDGE

Cutting Edge: Epstein-Barr Virus Transactivates the HERV-K18 Superantigen by Docking to the Human Complement Receptor 2 (CD21) on Primary B Cells[1]

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***EBV, a ubiquitous human herpesvirus, is the causative agent of infectious mononucleosis and is associated with many carcinomas. We have previously shown that the EBV latent genes* LMP-1 *and* LMP-2A *(for latent membrane proteins 1 and 2A), transactivate a human endogenous retrovirus (HERV), HERV-K18, in infected B lymphocytes. The envelope (Env) protein of HERV-K18 encodes a superantigen that strongly stimulates a large number of T cells. In this study we report that HERV-K18* env *is transactivated even earlier in the infection process, before the establishment of latency; namely, we found that EBV, through its interaction with its cellular receptor CD21, induces the HERV-K18* env *gene in resting B lymphocytes. This transactivation is direct and immediate, as up-regulation of transcripts can be detected within 30 min after EBV exposure. Thus, EBV binding to human CD21 on resting B cells triggers the expression of an endogenous superantigen. The biological significance of this superantigen expression for the EBV life cycle is discussed. The Journal of Immunology,* 2006, 177: 2056–2060.**

The interplay between a host and its pathogens forges an arms race that has been precariously kept at equilibrium by the force of evolution. Work in past decades unraveled the complex molecular mechanisms of such reciprocity with many emerging paradigms. EBV, one of the best-studied human viruses, offers an insight into these multifaceted interactions. A member of the γ-herpesvirus family, it has adapted successfully to infect and persist in 95% of the general human population, mostly with no apparent health repercussions. If delayed until adolescence, EBV infection may result in infectious mononucleosis, a self-limiting lymphoproliferative disease. Paradoxically, the herpesvirus preferentially infects and drives resting B lymphocytes into activated lymphoblasts with unlimited proliferative property and associated oncogenic potential in vitro (1–3). This apparent disparity may be largely attributed to the host's active immune surveillance. The breakdown of such defense, as seen in immunosuppressed patients, may result in the lethal posttransplant lymphoproliferative disease or other EBV^+ lymphomas (1). To evade the host's immune surveillance, the herpesvirus turns off the expression of all viral proteins and hides in the memory B cell compartment (2). It has been suggested that EBV mimics the normal pathway of B cell differentiation with its two latent membrane proteins (LMPs),[3] LMP-1 and LMP-2A, to gain access to the memory B cell compartment (2).

The discovery that EBV infection of B lymphocytes transactivates a superantigen encoded by the human endogenous retrovirus (HERV) HERV-K18 has added a new breadth to the complexity of host-pathogen interaction (4, 5). HERV-K18 belongs to a class of retroelements, HERVs, that retain much of the genomic organization of modern day exogenous retroviruses such as HIV (6). HERVs possess two long-terminal repeats that flank a region containing group-specific Ag (*gag*), polymerase and other enzymes, and envelope (*env*) genes and no longer make virions due to inactivating mutations accumulated through evolution. HERVs are believed to have arisen from retroviruses that integrated into the genome of an ancestral germline (6). Located on the first chromosome HERV-K18 (1q23.1-q24) sits on the first intron of the cellular gene *CD48* and has an opposite direction of transcription to that gene (7). Absent in New World monkeys, HERV-K18 is found in humans and Old World primates. Although most HERVs have no known biological functions, the Env protein encoded by HERV-K18 has a superantigen activity that is readily induced in lymphocytes upon EBV infection or human IFN-α treatment (4, 8). Superantigens are microbial proteins that overstimulate the immune system (9). They directly interact with the Vβ segment of the TCR, unlike conventional peptide Ags that are recognized by a particular third hypervariable region of the TCR that is different in every T cell clone. Thus, a vast number of T cells can mount a primary immune response to a superantigen. The physiological importance of a superantigen to the life

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[3] Abbreviations used in this paper: LMP, latent membrane protein; BAC, bacterial artificial chromosome; env, envelope; gag, group-specific antigen; hCD21, human CD21; HERV, human endogenous retrovirus; mIL-6, murine IL-6; MMTV, murine mammary tumor virus; qRT, quantitative reverse transcription.

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cycle of a virus has been studied in the case of the murine mammary tumor virus (MMTV). Without its superantigen, MMTV is severely compromised in viral replication and vertical transmission (10).

Efforts to understand how EBV transactivates the HERV-K18 superantigen revealed that LMP-2A and LMP-1 each contributes to the induction of the superantigen activity in latently infected cells in vitro (11). To further understand the biological significance of the superantigen, the status of the HERV-K18 *env* transcript was investigated during the earlier stages of the EBV infection process before the establishment of latency. CD21 (complement receptor 2), found mostly on B lymphocytes, is the cellular receptor that mediates the B cell tropism of EBV in humans (12). In addition to EBV, CD21 also interacts with three known ligands: the complement fragment C3d, CD23 (Fc receptor for IgE), and human IFN-α (13–16). Through the major EBV envelope glycoprotein, gp350, the herpesvirus binds to human CD21 (hCD21) and enters B cells by endocytosis (17). The interaction between gp350 and hCD21 triggers signal transduction pathways that culminate in the activation of NF-κB and the up-regulation of IL-6 transcript and protein (18–21). Subsequent to viral entry, latency is established in most infected B cells with the expression of LMP-1 and LMP-2A among other latent viral proteins (2).

Materials and Methods

Reagents, cell culture, and Abs

Cells were grown in RPMI with 10% FBS. Anti-hCD21 mAbs 171 and 1048 were from Dr. V. M. Holers (University of Colorado Health Sciences Center, Denver, CO). B-ly4 (BD Biosciences) and anti-murine IgM $F(ab')_2$ (Jackson ImmunoResearch Laboratories) were purchased. Recombinant gp350 was from Dr. A. Morgan (University of Bristol, Bristol, U.K.) and had <25 pg/ml endotoxin (18). The rgp350 was used at 1 μg/ml. EBV (B95.8) was generated and used as described (5).

Tonsil cell preparation

Lymphocytes were isolated by density gradient separation and resuspended in RPMI. Fine single cell suspensions (2×10^6) were used in each sample test.

Transgenic mice

Sequences of primer pairs for DNA PCR are available upon request. All animals were bred at the Tufts University (Boston, MA) Division of Laboratory Animal Medicine. The Institutional Animal Cared and Use Committee of Tufts University has approved all procedures.

Murine splenocyte isolation and B cell purification

To harvest splenocytes, RBCs were lysed with 1× PharmLyse (BD Biosciences) per instruction. To harvest purified B cells, IMag (direct magnet) mouse B lymphocyte enrichment set (BD Biosciences) was used on single cell suspensions from spleens per instruction. Splenocytes (2×10^6) or purified B cells (95–97% purity) were used in each sample test.

Plate-bound anti-hCD21 assay

Wells were coated with protein A (100 μg/ml) for 30 min at 37°C and then coated with anti-hCD21 mAbs (1 μg/ml) for an additional 30 min at 37°C.

Real-time quantitative reverse transcription (qRT)-PCR

Total RNA was isolated using the RNeasy mini kit (Qiagen). The cDNA was generated from 1 μg of total RNA using iScript reverse transcriptase (Bio-Rad). The TaqMan probe and primers specific for the read-through transcript of HERV-K18 were designed to recognize HERV-K18 *env* in human tonsil cells (6-FAM, minor groove binder probe, 5′-TAAGTCCTACAGACAAACTT-3′; forward primer, 5′-CCGCCTTTTGAGCAGAAGTATAAGA-3′; reverse primer, 5′-CAGTAATGGCAATGCTGGCTATG-3′). TaqMan probe and primers were designed to recognize HERV-K18 *env* in transgenic mice (6-FAM, minor groove binder probe, 5′-TTGATCCTTTAGGAATTTC-3′; forward primer, 5′-CCTAAAGGGAAAACTTGCCCCAA-3′; reverse primer, 5′-GCCACACATTCTTCCCAAACTAAA A-3′). TaqMan probe and primers for human and mouse IL-6, as well as 18S, were purchased from Applied Biosystems.

Results and Discussion

EBV, through gp350, transactivates HERV-K18 env in tonsil cells

To address the possibility that EBV transactivates HERV-K18 *env* in the host cell before viral gene expression, we first examined the effect of EBV incubation on human tonsil cells in a 30-min assay. Because EBV may carry LMP-1 and LMP-2A in its membrane as it buds off from the previous host cell, we chose a 30-miute assay to minimize potential carryover of membrane proteins (17). Using real-time qRT-PCR, we observed on average a 3-fold induction of the HERV-K18 *env* transcripts in these cells. In the same cDNA samples we found that IL-6 was similarly induced, serving as a positive control (Fig. 1*A*). To assess the possibility that the interaction between gp350 and hCD21 could account for this induction, we repeated the above tonsil cell experiments using rgp350 in a 2-h assay (18). We found that HERV-K18 *env* was up-regulated 2- to 3-fold and observed an induction of IL-6 (Fig. 1*B*). To further confirm this finding, we generated double transgenic mice that harbored both the hCD21 and HERV-K18 transgenes.

Generation of hCD21/HERV-K18 double transgenic mice

Because EBV and gp350 do not bind to mouse B cells (22), the generation of a hCD21 transgenic mouse allows us to study hCD21 function in isolation and provide the ideal negative control with nontransgenic littermates. Similarly, because mice do not have HERVs, the generation of HERV-K18 transgenic mice gives us the same advantage. Thus, we produced double transgenic mice expressing hCD21 and HERV-K18 from bacteria artificial chromosomes (BACs). The HERV-K18 transgenic mice have been described elsewhere (26). To ensure that the hCD21 transgene is expressed from its native human regulatory elements and promoters, we selected a BAC, RP11-35C1, that contains hCD21 flanked by complement receptor 1 (CD35) and a hypothetical protein for injection into fertilized

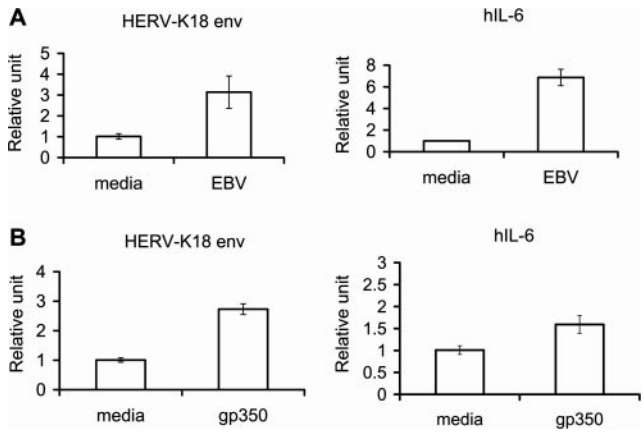

FIGURE 1. EBV and rgp350 up-regulate HERV-K18 *env* and IL-6 in tonsil cells. *A*, Tonsil cells were incubated with EBV for 30 min. Real-time qRT-PCR was performed to assess the relative HERV-K18 *env* and human IL-6 transcripts. On average, a 3-fold induction of HERV-K18 *env* and a 5-fold induction of IL-6 were observed. *B*, Tonsil cells were incubated with rgp350 for 2 h. On average, a 3-fold induction of HERV-K18 *env* and a 2-fold induction of IL-6 were observed. The target transcripts were normalized to the 18S ribosomal RNA transcripts in the same samples. Representative experiments were shown. Error bars are SE of triplicates from a single qRT-PCR assay.

FVB eggs. Three fertile founder mice (transgenic mice nos.18, 36, and 38) were selected by DNA PCR (Fig. 2*A*) and bred onto the C57BL/6 background using I-$A^{b-/-}$/DR4 transgenic mice. We further characterized the expression of hCD21 by flow cytometry. Transgenic mouse no.38 has the highest hCD21 expression, whereas nos.18 and 36 have comparable expressions (Fig. 2*B* and data not shown). Using B220 as a B cell marker, we observed that hCD21 is expressed mostly on B splenocytes, mirroring its expression pattern in humans (Fig. 2*B*). Because misexpression of hCD21 in mice can disrupt B cell development (23), we compared the splenic B cell profile between hCD21 transgenic mice and its littermates. We did not find a pronounced difference in the expression of mouse CD19, CD21/CD35, B220 (Fig. 2, *B* and *C*), or IgD (data not shown). Thus, the hCD21 transgenic mice are indistinguishable from their littermates, at least with regard to the particular parameters that were compared. After 5–10 generations of backcross, the most fertile line, transgenic mouse no.36, was crossed with the 14b HERV-K18 transgenic mice on the same background (Fig. 2*D*). We further confirmed that HERV-K18 *env* is inducible in our double transgenic mice by anti-IgM treatment of the purified B cells (Fig. 2*E*) (26).

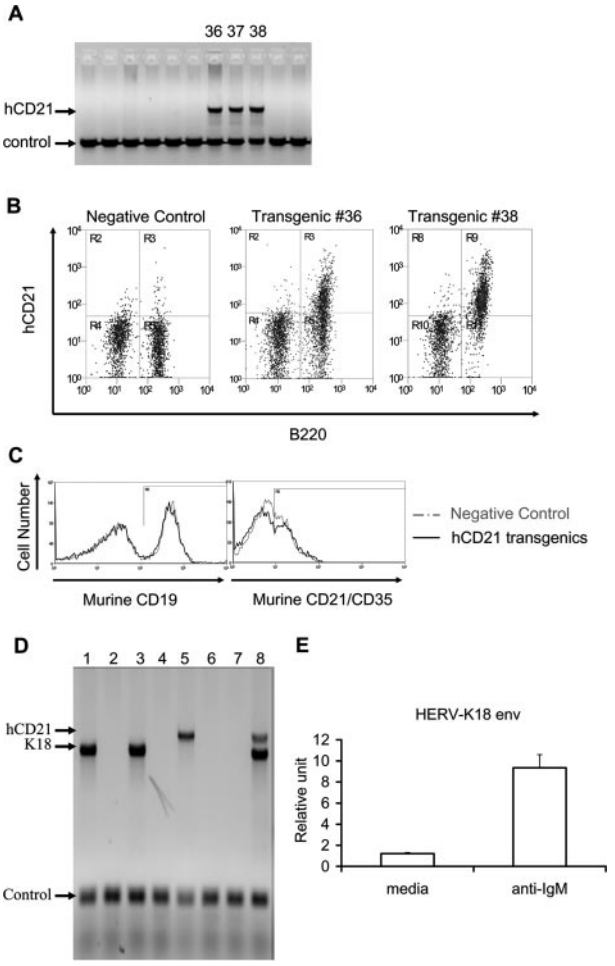

FIGURE 2. Generation and characterization of hCD21 and hCD21/HERV-K18 transgenic mice. *A*, gel showing the duplex PCR products of tail DNA used to screen for hCD21 transgenic mice. *Lanes 36*, *37*, and *38*, corresponding to transgenic mice nos.36, 37, and 38, respectively, showed integration of hCD21 BAC. *B*, FACS histogram of splenocytes from negative control and transgenic mice nos.36 and 38 stained using hCD21 and B220 to mark B cells. *C*, Overlay FACS histograms of splenocytes staining from a negative control and transgenic mouse no.36 splenocytes, showing similar mCD19 and mCD21/mCD35 expression patterns. *D*, gel showing the triplex PCR products of tail DNA used to screen for hCD21/HERV-K18 transgenic mice. *Lane 8* is a hCD21/HERV-K18 mouse, and *lanes 1* and *2* are the HERV-K18 littermates. *E*, qRT-PCR data showing that HERV-K18 *env* was inducible by anti-IgM in the B cells of a hCD21/HERV-K18 transgenic mouse.

EBV, through gp350, transactivates HERV-K18 env *and murine IL-6 (mIL-6) in B cells of hCD21/HERV-K18 double transgenic mice*

To assess whether we can recapitulate our observation made in human tonsil cells in the double transgenic mice, we incubated EBV with the splenocytes of hCD21/HERV-K18 transgenic mice in a 2-h assay. On average, we observed a 3.5-fold transactivation of HERV-K18 *env*. In contrast, experiments performed using splenocytes from the littermates exhibited a decrease in HERV-K18 *env* (Fig. 3*A*). To test the possibility that signal initiated from hCD21 can transactivate mIL-6, we performed qRT-PCR for mIL-6 on the same cDNA samples. Interestingly, mIL-6 was induced equally well (Fig. 3*A*) and followed kinetics of transactivation similar to that of HERV-K18 *env* (data not shown). To investigate whether gp350 mediates the observed induction, we repeated the same experiment on purified B cells with rgp350. We found that HERV-K18 *env* is induced in the B cells of hCD21/HERV-K18 transgenic mice but not in those of HERV-K18 transgenic littermates (Fig. 3*B*). We observed a similar trend of mIL-6 induction in the same cDNA samples (Fig. 3*B*).

Human CD21 cross-linking is sufficient to transactivate HERV-K18 env *and mIL-6 in B cells of hCD21/HERV-K18 transgenic mice*

To further confirm that hCD21 signaling is responsible for HERV-K18 *env* and IL-6 transactivation, we incubated purified B cells of hCD21/HERV-K18 and HERV-K18 mice with plate-bound anti-hCD21 mAbs. Similar to gp350 of EBV,

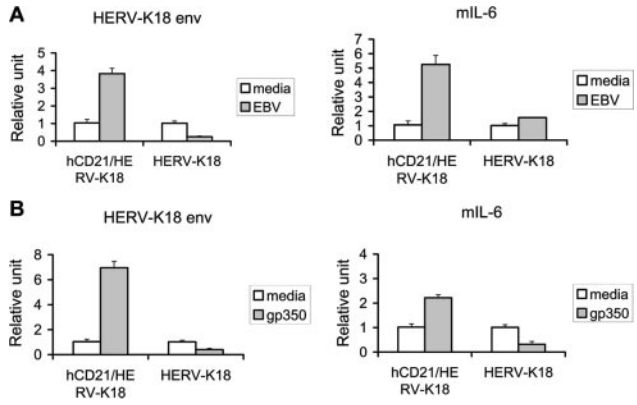

FIGURE 3. EBV and rgp350 up-regulate HERV-K18 *env* and mIL-6 in hCD21/HERV-K18 splenocytes. Splenocytes from hCD21/HERV-K18 transgenic mice and HERV-K18 littermates were incubated with EBV for 2 h. Real-time qRT-PCR was performed to assess the relative HERV-K18 *env* and mIL-6 transcripts. *A*, In comparison to samples without EBV incubation, on average a 3-fold induction of HERV-K18 *env* and a 5-fold induction of mIL-6 were observed in hCD21/HERV-K18 splenocytes. No significant inductions of either HERV-K18 *env* or mIL-6 were observed in HERV-K18 splenocytes. *B*, Purified B cells from hCD21/HERV-K18 transgenic mice and HERV-K18 littermates were incubated with rgp350 for 2 h. On average, a 5-fold induction of HERV-K18 *env* and a 3-fold induction of mIL-6 were observed in hCD21/HERV-K18 B cells. No significant inductions of either HERV-K18 *env* or mIL-6 were observed in HERV-K18 B cells. Representative experiments were shown. Error bars are SE.

clones 171, 1048, and B-ly4 recognize hCD21 but not mCD21 (24). In a 2-h assay, we found up-regulation of HERV-K18 *env* and mIL-6 only in mouse B cells expressing hCD21 (Fig. 4). In contrast to plate-bound mAbs, soluble anti-hCD21 mAbs did not exhibit significant HERV-K18 *env* or IL-6 transactivation (data not shown).

PKCs and PTKs mediate HERV-K18 env and IL-6 transactivation by EBV in tonsil cells

To delineate signaling mechanisms for HERV-K18 *env* transactivation by EBV, we tested small molecule inhibitors aiming at various signaling pathways in tonsil cells. We found that bisindolylmaleimide (protein kinase C inhibitor; 5 μM) and herbimycin A (protein tyrosine kinase inhibitor; 8.7 μM) efficiently suppressed EBV induction of HERV-K18 *env* in 1 h (Fig. 5*A*), whereas LY294002 (PI-3K inhibitor; 40 μM) showed very little inhibition. AG490 (JAK2 inhibitor; 50 μM) was not inhibitory at all and serves as a negative control. Our data of HERV-K18 *env* inhibition are similar to those seen with IL-6 inhibition in both our assays (Fig. 5*B*) and the published reports (18, 20). This study suggests that HERV-K18 *env* and IL-6 share similar signal pathway(s) in our system.

In conclusion, we have shown that HERV-K18 *env* and IL-6 are controlled similarly at the transcriptional level, perhaps through an evolutionarily conserved pathway. With anti-hCD21 Abs, we demonstrated that ligands other than the EBV gp350 could transactivate IL-6 and HERV-K18 *env*. IL-6 is a known B cell growth factor and may provide an autocrine survival advantage to infected cells. Similarly, we postulate that the HERV-K18 superantigen may confer a survival advantage.

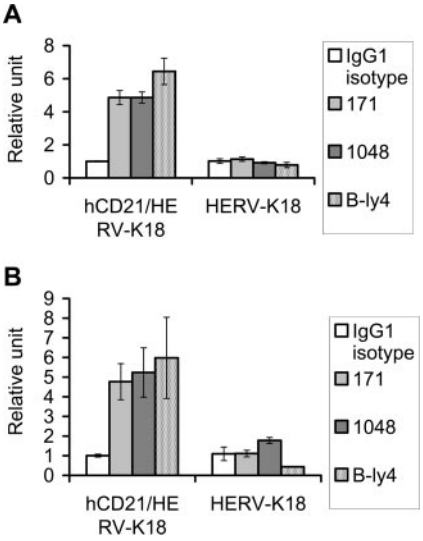

FIGURE 4. hCD21 ligation is sufficient to transactivate both HERV-K18 *env* and mIL-6 in hCD21/HERV-K18 B cells. Purified B cells from hCD21/HERV-K18 mice and HERV-K18 littermates were incubated with protein A anchored, anti-hCD21 mAbs for 2 h. Real-time qRT-PCR was performed to assess the relative HERV-K18 *env* and IL-6 transcripts. *A*, Relative to the protein A-anchored isotype control, a 5–6-fold induction of HERV-K18 *env* was observed with hCD21 ligation in the B cells of hCD21/HERV-K18 transgenic mice. No significant induction was observed in the B cells of the HERV-K18 littermates. *B*, In the same cDNA samples, on average a 4–6-fold induction of HERV-K18 *env* was observed in B cells of hCD21/HERV-K18 transgenic mice, but not in the B cells of HERV-K18 littermates. Representative experiments were shown. Error bars are SE. IgG1 is the isotype control.

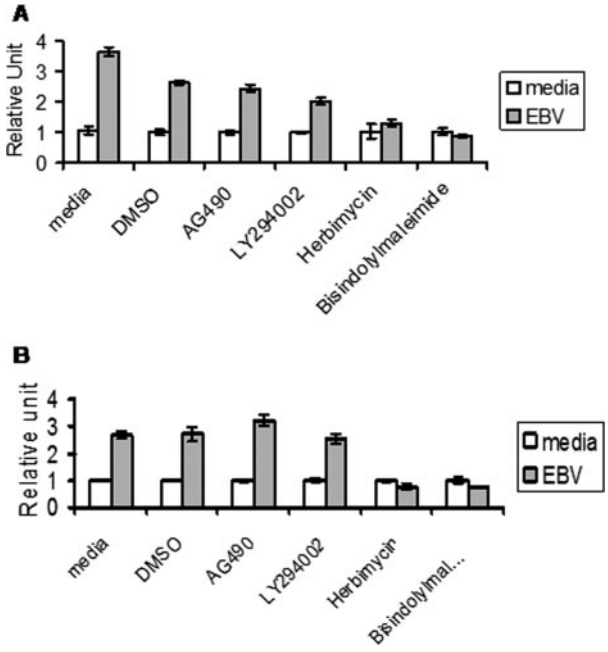

FIGURE 5. Protien kinase C and protein tyrosine kinase mediate HERV-K18 *env* and IL-6 transactivation by EBV. Tonsil cells were pretreated with small molecule inhibitors, dissolved in DMSO for 45 min, and then stimulated with EBV for 1 h. Real-time qRT-PCR was performed on the samples. Bisindolylmaleimide and herbimycin A efficiently suppressed EBV induction of both HERV-K18 *env* (*A*) and IL-6 (*B*), whereas LY294002 exhibited very little inhibition. AG490, serving as a negative control, did not show inhibition. Representative experiments were shown. Error bars are SE.

We have demonstrated previously with our collaborators that IFN-α transactivates HERV-K18 *env* in PBMCs (8). Interestingly, IFN-α has been shown to bind to hCD21, thereby inhibiting EBV from adsorption and capping (13, 15). Because we have linked gp350 binding of hCD21 to HERV-K18 and IL-6 up-regulation, we speculate that hCD21 may potentially mediate the induction by IFN-α of HERV-K18 *env* and possibly IL-6. This parallel is particularly fitting, because both gp350 and IFN-α are not known to cross-link CD21. Chronic administration of IFN-α is associated with autoimmune-like side effects. It will be interesting to see whether the transactivation of HERV-K18 *env* or IL-6 plays a role in causing these side effects.

Previously, we showed that the EBV-associated superantigen HERV-K18 *env* was induced in latently infected B cells (4, 5). Subsequently, we demonstrated that two EBV-encoded proteins, LMP-2A and LMP-1, are each responsible for transactivating HERV-K18 *env* during latency (11). In the present study we have shown that HERV-K18 *env* is transactivated as early as when EBV first docks to hCD21 on its host cell, before the establishment of latency. Hence, we have defined a novel time line in the life cycle of EBV during which the superantigen may function. The exact functional consequence of the superantigen activity remains to be elucidated. MMTV encodes a superantigen that provides T cell help to infected B lymphocytes, enabling viral transmission from the gut to mammary tissues (25). This process is critically important for successful infection and vertical transmission. We hypothesize that the EBV-induced superantigen activity provides a survival advantage through T cell help as well. Because EBV infects humans very efficiently, often at a very low titer in saliva, we speculate that HERV-K18 superantigen activity initiated at the onset of infection may aid

EBV in processes such as viral entry, viral replication, or viral gene transcription.

Acknowledgments

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Disclosures

The authors have no financial conflict of interest.

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