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Sensing of latent EBV infection through exosomal transfer of 5′pppRNA

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Complex interactions between DNA herpesviruses and host factors determine the establishment of a life-long asymptomatic latent infection. The lymphotropic Epstein–Barr virus (EBV) seems to avoid recognition by innate sensors despite massive transcription of immunostimulatory small RNAs (EBV-EBERs). Here we demonstrate that in latently infected B cells, EBER1 transcripts interact with the lupus antigen (La) ribonucleoprotein, avoiding cytoplasmic RNA sensors. However, in coculture experiments we observed that latent-infected cells trigger antiviral immunity in dendritic cells (DCs) through selective release and transfer of RNA via exosomes. In ex vivo tonsillar cultures, we observed that EBER1-loaded exosomes are preferentially captured and internalized by human plasmacytoid DCs (pDCs) that express the TIM1 phosphatidylserine receptor, a known viral- and exosomal target. Using an EBER-deficient EBV strain, enzymatic removal of 5′ppp, in vitro transcripts, and coculture experiments, we established that 5′pppEBER1 transfer via exosomes drives antiviral immunity in nonpermissive DCs. Lupus erythematosus patients suffer from elevated EBV load and activated antiviral immunity, in particular in skin lesions that are infiltrated with pDCs. We detected high levels of EBER1 RNA in such skin lesions, as well as EBV-microRNAs, but no intact EBV-DNA, linking non–cell-autonomous EBER1 presence with skin inflammation in predisposed individuals. Collectively, our studies indicate that virus-modified exosomes have a physiological role in the host–pathogen stand-off and may promote inflammatory disease.

exosomes | EBV-EBER1 | innate sensing | dendritic cells | skin inflammation

Viral nucleic acids are among the foreign molecules with pathogen-associated molecular patterns (PAMPs) that are recognized by innate immune host-sensors in the cytosol (1). Some RNA viruses establish a chronic productive infection: for example, hepatitis C virus (HCV), which evolved strategies to escape from innate immune sensors contributing to its pathophysiology (2). Nevertheless HCV, an enveloped RNA virus, can be recognized by sensory plasmacytoid dendritic cells (pDCs) (3). More recently, Hepatitis A virus, a nonenveloped RNA virus, was shown to hijack a host membrane, presumably to escape neutralizing antibodies while facilitating sensing by pDCs (4). How DNA viruses, and in particular latent herpes viruses, are recognized by the innate immune system and whether this causes or protects from pathogenesis remains unclear (5).

Chronic viral infections can be asymptomatic and only cause pathogenesis in immune-suppressed or genetically predisposed individuals, a prime example being infection by the large DNA herpesvirus Epstein–Barr virus (EBV) (6). Although there is no accurate animal model available, studies in human tissue and peripheral blood showed that EBV has a strong B-cell tropism and evolved a complex latency strategy to minimize transcriptional activity and avoid immune detection. Still, during persistence, EBV produces up to $10^{5–6}$ copies of polymerase III (Pol III)-transcribed noncoding RNAs (EBERs) per infected B cell (7). EBERs consist of EBER1 and EBER2 molecules, although in latent-infected cells, EBER1 levels are generally higher. EBER1 expression is reduced in reactivated cells, implying a function in maintaining EBV latency, although in vivo EBER1 function remains unclear (8). EBER1 and EBER2 form stem-loop structures by intramolecular base pairing, which enables interaction with several cellular proteins.

Significance

Increasing evidence suggests that the exosomal messenger pathway warns neighboring cells against cellular stress and infection. Recent studies have shown that viruses and cancer cells exploit exosomes to transmit functional RNAs. Our studies reveal that a viral small RNA signal for innate immunity Epstein–Barr virus (EBV)-EBER1 is produced by latent EBV-infected B cells and recognized by noninfected dendritic cells activating an inflammatory response. We detected high amounts of EBV-EBER1 transcripts and EBV-microRNAs in inflamed skin lesions of autoimmune patients that are infiltrated with dendritic cells. Importantly, we found virtually no EBV-DNA present in these tissues, suggesting that continuous cell–cell EBER1 transmission via exosomes occurs in humans. We propose that innate sensing of latent EBV in predisposed individuals may be more harmful than previously thought.

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Both double-stranded loops and the uncapped 5′-triphosphate terminus of EBERs serve as ligands for pathogen recognition receptors (PRRs) (9–12). Because infected individuals (estimated at 90% of the world population) generally have no symptoms, it seems that EBER production in healthy individuals elude cell-intrinsic danger signaling. Currently, the recognition of herpesviruses during latent infection remains poorly understood (5).

Toll-like receptors (TLRs) -3, -7, and -8 are PRRs expressed in sensory dendritic cells that detect exogenous double- and single-stranded viral RNA in endosomes. Because the binding domains of TLRs face toward the lumen of endosomes, recognition of self-RNAs in the cytoplasm is avoided (13). Moreover, mRNAs are normally 5′-"capped" and bound by poly-A binding proteins, whereas tRNAs are enzymatically modified and ribosomal RNAs are masked as ribonucleoprotein complexes, preventing them from being recognized by cytosolic PRRs. Retinoic acid-inducible gene I (RIG-I) (14), IFN-induced proteins with tetratricopeptide repeats (IFITs) (15), and the double-stranded RNA-dependent protein kinase (PKR) can recognize 5′triphosphates (5′ppp)-bearing viral RNA in the cytoplasm (16). Antiviral signaling is also induced by modified self-RNAs under physiological stress conditions (17–19). Removal of proinflammatory self-RNAs, while maintaining the ability to selectively recognize virus-produced RNAs in the cell's cytoplasm, is essential for proper regulation of antiviral response. Indeed, chronic activation of antiviral immunity by erroneous detection of extracellular RNA drives autoimmunity in predisposed individuals (20). Although binding of proinflammatory EBERs to cytoplasmic sensors has been reported in vitro (9–12), there is little evidence that this may occur in latently infected B cells in a physiologically relevant context (21), but there are multiple indications that EBERs are secreted from infected cells (9, 22, 23).

Here we determined a physiological effect of secreted EBER1 via exosomes, a subtype of extracellular vesicles of endosomal origin. We demonstrate that latently infected B cells sort 5′pppEBER1 molecules into exosomes that are taken up by DCs and trigger antiviral immunity. In a chronic setting, innate sensing of latent EBV by pDCs may pose an environmental risk factor for developing and driving nonresolving organ inflammation.

Results

Exosomes from Latently EBV-Infected Cells Induce Antiviral Immunity in Primary DCs. We showed previously that latency III EBV-infected B cells influence gene expression in neighboring monocyte-derived DCs through exosome-mediated transfer of functional viral microRNAs (miRNAs) (24).

We wished to gain a global insight into the genes and pathways affected in DCs upon internalization of exosomes released by EBV-infected B cells. We purified exosomes from EBV-driven (latency III) lymphoblastoid cell lines (EBV$^+$ LCLs) and noninfected (EBV$^-$) B-cell lymphoma control cells (BJAB) that produce similar amounts of exosomes (25) ("LCL exosomes" and "Ctrl exosomes," respectively). We incubated primary DCs with LCL and Ctrl exosomes and analyzed the gene expression profile of DCs with high-density arrays after 18 h of incubation (Fig. 1*A*). Gene ontology (GO) analysis (DAVID bioinformatics tool) showed that immune, defense, and inflammatory responses and "response to wounding" were the top four generally modulated processes in exosome-stimulated conditions (Fig. 1 *B* and *C*). Control exosomes also affected

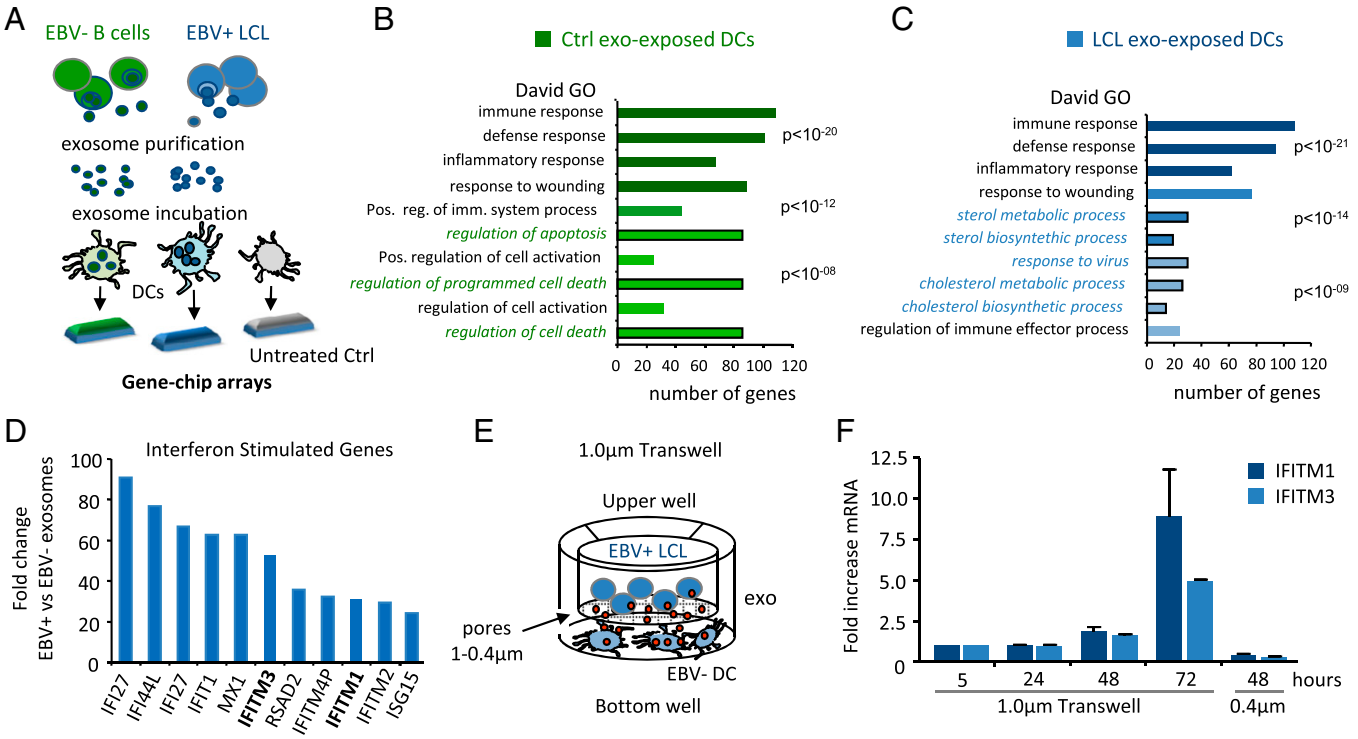

Fig. 1. Exosomes released by latent EBV-infected LCLs trigger antiviral immunity in DCs. (*A*) Schematic representation of experimental design: primary DCs were incubated with exosomes derived from EBV$^+$ LCLs or EBV$^-$ control B cells (BJAB), and a gene-expression profile was performed using high-density arrays after 18 h of incubation. (*B* and *C*) GO analysis performed with DAVID showing the top modulated pathways in DCs exposed to EBV$^-$ control B-cell (*B*) and EBV$^+$ LCL (*C*) exosomes compared with untreated DCs. (*D*) Fold-difference of IFN-stimulated genes in DCs exposed to LCL exosomes compared with control exosomes. (*E*) Schematic representation of a transwell coculture system: exosome-producing B cells (upper compartment) and recipient DCs (lower compartment) are separated by a 1-μm pore size membrane, which allows the passage of exosomes. (*F*) Quantitative PCR (qPCR) for *IFITM1* and *IFITM3* in DCs at indicated time points during coculture using a transwell with either 1- or 0.4-μm membrane pores. Transcript levels are normalized to *GAPDH* and expressed as fold-increase relative to $t = 0$.

apoptosis-related pathways, but to a lesser extent. In contrast, incubation with LCL exosomes induced antiviral response-related pathways including sterol metabolic and biosynthetic processes in the DCs. Sterol-associated biochemistry was recently implicated in type I IFN-driven antiviral immunity induced by viral infection (26). In fact, type I IFN-stimulated genes (ISGs) were among the most strongly induced genes in DCs exposed to LCL exosomes. Among the most elevated genes in DCs exposed to LCL exosomes compared with Ctrl exosomes were interferon induced transmembrane protein 1 (*IFITM1*), which inhibits the entry of viruses to the cytoplasm, *IFITM3*, which disrupts intracellular cholesterol homeostasis (Fig. 1*D*), and the transcription factor *STAT1* (27). Highly similar differences in the induction of *IFITM1* and *IFITM3* were observed when comparing DCs exposed to LCL exosomes or CD40L-stimulated primary B cell exosomes (Fig. S1 *A* and *B*). We conclude that LCLs exosomes but not control exosomes from EBV$^-$ B cells induce an antiviral transcription program in noninfected DCs.

To investigate whether the continuous release of exosomes in culture could reproduce the type I IFN-response signature induced by purified exosomes, we cocultured latently infected LCLs with primary DCs and measured *IFITM1* and *IFITM3* mRNA levels as markers for antiviral response activation (Fig. 1*E*). We detected a steady increase in *IFITM1* and *IFITM3* mRNA levels reaching twofold induction after 48 h and eightfold induction after 72 h (Fig. 1*F*), indicative of antiviral response activation. Interestingly, when we decrease exosome-transfer by separating the LCLs from DCs with a 0.4-μm porous membrane (instead of an exosome-permissive 1.0-μm membrane), we did not observe any induction of *IFITM1* and *IFITM3* after 48 h, suggesting that soluble factors are not involved in early activation (Fig. 1*F* and Fig. S1*C*). Thus, latently EBV-infected B cells secrete exosomes that can induce antiviral, proinflammatory behavior in sensory immune cells.

Latent EBV-Infected Exosomes Do Not Display an Antiviral Proteome Signature. Recent studies in IFN-exposed DCs suggested that exosomes incorporate and transfer antiviral proteins (28). To investigate the possibility that latent EBV-infected cells release antiviral proteins via exosomes, we performed label-free mass spectrometric proteomics on LCLs whose proliferation is EBV-driven and, among others, CD40L-stimulated uninfected B cells with identical genetic background as a physiologically relevant control (Dataset S1). Differential analysis comparing CD40L-driven EBV$^-$ primary B cells (prim B-cells) with LCL cells driven by latent EBV, and subsequent GO mining of 113 proteins that were significantly more abundant in LCLs ($P < 0.05$) showed enrichment of the term "response to virus" (corrected $P < 0.05$) (Fig. S2 and Dataset S1). Proteins linked to this term were encompassed within a major cluster of protein–protein associations retrieved from the STRING database (29) (Fig. 2*A* and Fig. S2). Moreover, the majority of the proteins in this cluster are linked to type I IFN signaling and function. When comparing mass-spectral counts for selected proteins in two distinct LCL lines versus CD40L-driven and EBV$^-$ control B cells (BJAB) (Fig. 2*B*), the counts showed a consistent difference, indicating that the antiviral signature is related to EBV infection in human B cells.

In parallel, we analyzed the proteome of exosomes released by EBV-driven LCL and exosomes from CD40L-driven B cells, all purified by ultracentrifugation on a sucrose cushion, as described previously (30). We detected 3,000+ proteins in the exosomes (Dataset S2). Interestingly, apart from immune-response terms, proteins enriched in the LCL exosomes relative to their EBV$^-$ counterparts (144 differential proteins in total, $P < 0.05$) were associated with "integrin-mediated signaling" and cell-adhesion terms (Fig. 2*C* and Fig. S3). Supportively, we identified characteristic EBV-LMP1 (latent membrane protein 1)–induced proteins enriched in LCL exosomes, including EBI2/GPR183, STAT1, and CD48/BLAST-1 proteins (Fig. 2*D*), consistent with a latent EBV signature. The most enriched antiviral protein in LCL exosomes was IFITM3, consistent with its subcellular localization at endosomal membranes (31). Generally, the spectral counts for antiviral proteins in both the LCL exosomes and EBV$^-$ B-cell exosomes were low. We conclude that the restricted antiviral protein signature in EBV-driven LCL is not selectively conferred to exosomes. This finding is distinct from exosomes released by cells that are exposed to IFN-α, which carry an elaborate antiviral proteome (28).

EBER1 Sorting and Transmission via Exosomes Induces Antiviral Immunity in Primary DCs. We previously found that the small RNA content of LCL exosomes includes functional Pol II-transcribed EBV-miRNAs (25). Bioanalyzer analysis of purified exosomal RNA indicated that latently infected LCLs and (CD40L-stimulated) EBV$^-$ primary B cells produce exosomes enriched in small RNA species, ranging from 15 to 200 nucleotides. RNA-seq analysis showed that exosomes carry multiple RNA classes, although their

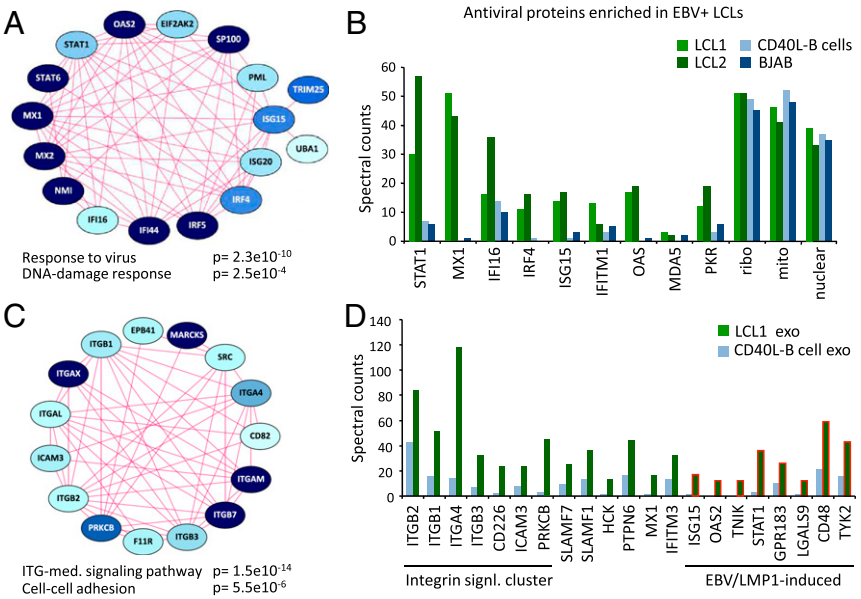

Fig. 2. Latent EBV-infected cells display an antiviral proteomic signature that is not conveyed to their exosomes. (*A* and *B*) Proteomics analysis on EBV-driven LCLs compared with CD40L-stimulated B cells reveals enrichment in the pathway "response to virus" (Fig. S2) and "DNA-damage response," with a dominant cluster of 17 antiviral proteins (*A*). Spectral counts of LCL cell lines, CD40L-stimulated B cells and BJAB cells showing association of the antiviral protein signature with EBV infection. Ribo: 40S ribosomal protein S3; mito: Stress-70 protein, mitochondrial; nuclear: Splicing factor 3B subunit 1 (*B*). (*C* and *D*) Proteomics analysis of exosomes from EBV-driven LCLs compared with CD40L-stimulated B cells (Fig. S3) reveals enrichment of "integrin-mediated signaling pathway" and "cell–cell adhesion" terms (*C*). Spectral counts showing enrichment of integrin signaling proteins and EBV/LMP1-induced proteins in LCL exosomes (*D*).

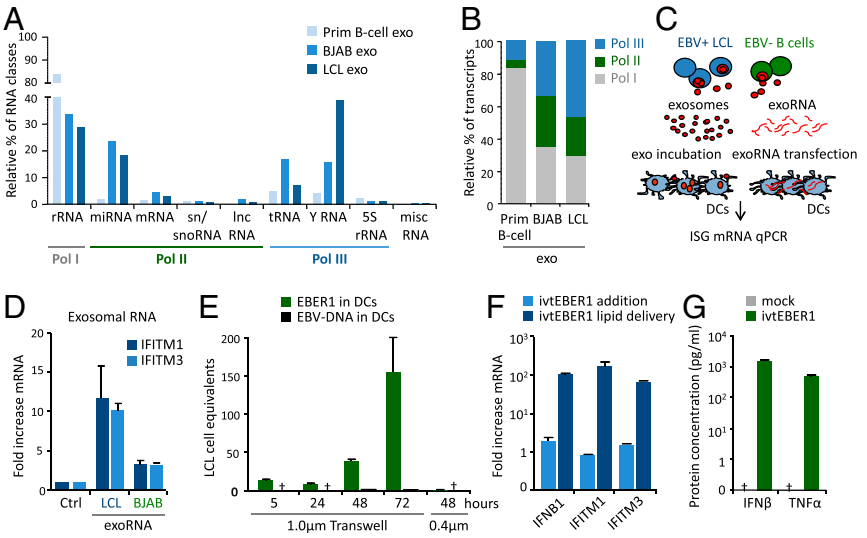

Fig. 3. Exosome-mediated transfer of EBER1 triggers an antiviral response in recipient DCs. (*A* and *B*) Small RNA class distribution in exosomes from EBV$^+$ LCLs and EBV$^-$ BJAB and primary B cells. Data are expressed as percentage relative to the total amount of sequencing reads (*A*). Relative percentage of Pol I, II, and III transcripts in LCL and control exosomes (*B*). (*C* and *D*) DCs were either incubated with exosomes from EBV$^+$/EBV$^-$ cells or transfected with matching amounts of exosomal RNA (exoRNA), and the expression of ISGs was assessed by qPCR after 18 h of incubation (*C*). Exosomal RNA from EBV$^+$ LCLs, but not from EBV$^-$ BJAB cells induced *IFITM1* and *IFITM3* expression (*D*). (*E*) qPCR analysis of EBER1 RNA and EBV-DNA in DCs at specific time points during the coculture period, using transwells with 1- or 0.4-μm membrane pores. qPCR data are expressed as EBV-infected LCL cell equivalents. (*F*) qPCR analysis of *IFITM1*, *IFITM3*, and *IFNB1* in DCs upon transfection or direct addition of 5′pppEBER1. Transcript levels are normalized to *GAPDH* and expressed as fold-increase relative to experimental controls. (*G*) IFN-β and TNF-α production by DCs transfected with 5′pppEBER1; protein concentration was assessed by ELISA on culture supernatants.

relative distribution is different (Fig. 3*A*). We classified the RNA sequences in Pol I, II, and III transcripts and found that almost 50% of all transcripts were Pol III-transcribed in LCL exosomes, against the 12% in the EBV$^-$ primary B-cell–derived counterparts (Fig. 3*B*). This observation is consistent with elevated levels of BDP1 and TFIIIC transcription factors in latently EBV-infected LCLs that stimulate host Pol III transcription (32).

Depending on their sequence, structure, and modifications, small RNAs may or may not trigger specialized viral sensors that recognize PAMPs, such as small double-stranded RNA loops or a 5′ppp moiety (14). To investigate whether exosome-incorporated small RNAs trigger viral sensors, we purified and transfected exosomal RNA (exoRNA) directly into DCs (Fig. 3*C*). Interestingly, exoRNA from latently infected LCLs strongly induced *IFITM1* and *IFITM3* transcription, compared with exoRNA from EBV$^-$ control (BJAB) cells (Fig. 3*D*), in agreement with the outcome of the array analysis in Fig. 1. Incubation of DCs with exoRNA or matched amounts of intact exosomes determined a similar fold-induction of *IFITM1* and *IFITM3* (Fig. S4*A*), suggesting that the small RNA content in LCL exosomes is modified by latent EBV infection and contains a trigger for antiviral gene expression.

EBV-encoded EBERs have proinflammatory properties and may reach up to 10^6 copies per latently infected B cell, with EBER1 being present at 10-fold higher levels than EBER2 (7). This finding is consistent with our small RNAseq analysis, indicating that EBER1-mapped reads outnumber those derived from EBER2 (Fig. S4*B*). To explore whether EBV$^+$ LCL exosomes package full-length EBERs while avoiding unwanted codetection of EBV-DNA, we designed stem-loop RT-PCR assays. Full-length EBER1, but not EBER2, was detectable in recipient DCs after coculture with LCLs (Fig. S4*C*). EBER1 RNA levels increased in the recipient cells over time, suggesting that exogenously delivered EBER1 molecules are not immediately degraded (Fig. 3*E*). We ruled out the possibility that DCs were initially or subsequently infected with EBV, as EBV-DNA was not detected by DNA-PCR, indicating that the EBER1 molecules were most likely of exogenous origin. We deemed transfer via EBER1–protein complexes released by dying cells unlikely, because use of small (0.4 μm) pore size that obstructs LCL exosome passage blocked all EBER1 transfer (Fig. 3*E*).

To test whether EBER1 is detected by physiologically relevant cells expressing viral sensors (33), we generated EBER1 molecules by in vitro transcription (ivtEBER1) and introduced them into primary DCs by lipid transfection. Both *IFITM1* and *IFITM3*, as well as interferon beta 1 (*IFNB1*) mRNA levels, were dramatically induced upon lipid delivery of EBER1 transcripts (Fig. 3*F*), whereas this was not observed by adding identical amounts of EBER1 directly to the culture dish. Finally, ELISAs demonstrated that EBER1 delivery stimulates the production of the inflammatory cytokines IFN-β and TNF-α by DCs (Fig. 3*G*). Thus, delivery of exogenous 5′pppEBER1 via lipid vesicles triggers antiviral gene expression and inflammatory cytokines production in primary DCs.

EBER1 Sensing Is Dependent upon Its 5′Triphosphate Moiety. Sensor/PRR activation relies on the RNA structure, posttranscriptional modifications, protein-binding affinity, and subcellular localization of the activating RNA (1). We investigated 5′pppEBER1 (Fig. 4*A*) recognition in Hek293 cells overexpressing TLR9, TLR3, or induced RIG-I, by measuring IFN-β or NF-κB promoter activation with luciferase-reporters. IFN-β and NF-κB promoters were activated by 100 ng of in vitro transcribed 5′pppEBER1 in TLR3- and RIG-I–expressing cells, but not in TLR9-expressing or parental Hek cells (Fig. 4*B*). To formally establish that exosome-mediated transfer of EBER1 activates an antiviral response in noninfected DCs, we isolated exosomes from LCLs driven by an EBER-deficient mutant EBV strain (34). We performed microarray analysis of DCs that were incubated with EBER1-deficient (EB.KO) or EBER1-containing (wild-type) LCL exosomes. In a direct comparison we found that EBER1-containing exosomes were superior in their capacity to activate antiviral genes compared with EBER1-deficient LCL exosomes (Fig. 4*C*).

To investigate whether the 5′ppp moiety of EBER1 serves as a PAMP in recipient DCs, we isolated exosomal RNA and treated this with calf intestinal phosphatase (CIP) to remove the phosphate groups. Subsequently we transfected CIP-treated and untreated exoRNA into DCs and measured ISG expression (Fig. 4*D*). Strikingly, CIP treatment completely impeded ISG induction (Fig. 4 *E*–*H*). EBER1 levels were nevertheless similar in DCs transfected with CIP-treated and nontreated exoRNA (Fig. 4*D*), excluding variation in intracellular levels as an explanation for the observed differences. Moreover, the exoRNA from EBER1-deficient LCLs induced ISG expression to a much lower extent compared with the exoRNA from wild-type EBV$^+$ LCLs, suggesting that EBER1 is a major factor in the IFN response (Fig. 4 *E*–*H*). To confirm that exosome-transmitted EBER1 causes this effect, we electroporated 5′pppEBER1 transcripts into exosomes from EBER$^-$ control cells. We found that 5′pppEBER1 addition to EBER$^-$ exosomes fully recovered the induction of IFN response (Fig. S4*D*). These results,

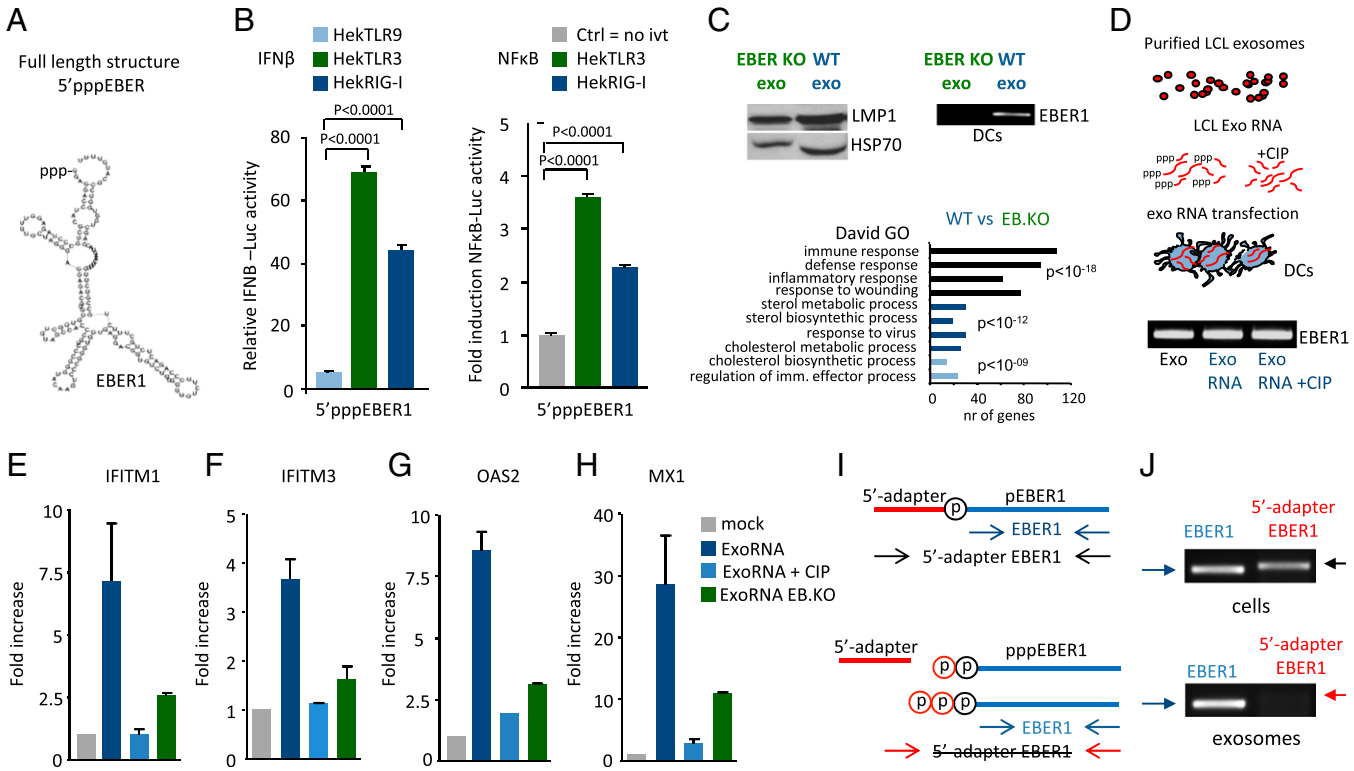

Fig. 4. The 5′triphosphate moiety of EBER1 is required for antiviral response activation. (*A*) Predicted secondary structure of EBER1 (RNAfold web server). (*B*) IFN-β and NF-κB promoter activation (assessed with luciferase reporters) in response to 5′pppEBER1 (ivt) transfection in Hek293 cells expressing TLR9, TLR3, or RIG-I (cells were "primed" with rhIFN-α). Firefly luciferase activity is normalized to Gaussia luciferase activity and data are expressed relative to control. (*C*) Western blot analysis of LMP1 and Hsp70 in exosomes isolated from LCLs infected with EBER-deficient mutant (EB.KO) or wild-type (wt) EBV (*Upper Left*). DCs were incubated with EBER1-deficient or EBER1-containing exosomes and the EBER1 levels were assessed by qPCR (*Upper Right*). Microarray analysis (*Lower*) shows that antiviral genes are strongly overrepresented in DCs exposed to wild-type exosomes compared with DCs incubated with EBER1-deficient exosomes. (*D–H*) Exosomal RNA was incubated with CIP to remove the phosphate moieties, and DCs were transfected with CIP-treated or untreated exosomal RNA. EBER1 levels were comparable in all experimental conditions (*D*). qPCR analysis shows that treatment with CIP completely abrogates the ability of exosomal RNA to induce ISGs. Transcript levels are normalized to *GAPDH* and expressed as fold-increase relative to mock control (*E–H*). (*I* and *J*) LCL cellular and exosomal RNA were incubated with adapters that can only ligate 5′monophosphate RNAs; qPCR was performed using primers specific for the 5′adapter and 3′EBER1 (*I*). Full-length EBER1, but not 5′adapter ligated-EBER1, is detectable in LCL exosomes (*J*).

combined, suggest that EBER1 molecules in exosomes bear a 5′ppp motif and induce antiviral genes in DCs. To corroborate this finding, we ligated adapters to 5′monophosphate-bearing small RNA molecules and performed RT-PCR with a 5′adapter and 3′ EBER1 specific primer set (Fig. 4*I*). Although we readily detected intracellular EBER1, we did not detect adapter-ligated EBER1 in the exosomes, yet with conventional EBER1 primers we confirmed the presence of EBER1 (Fig. 4*J*). Thus, the exosomal pool of EBER1 harbors minimally a di- or triphosphate group that both function as a viral PAMP in cultured monocyte-derived DCs.

Cytoplasmic 5′pppEBER1 in Latently Infected B Cells Evades Viral Sensors by Association with Lupus Antigen Protein. Because EBV-EBER1 fails to trigger inflammatory cytokine production in latently infected LCLs, we hypothesized that these cells may lack viral sensors to detect them. We profiled LCLs and primary B cells by RT-PCR for nine prominent sensors and found that they were all expressed at the transcriptional level (Fig. 5*A*). RIG-I and PKR, both of which can sense 5′pppRNA, were also detected at the protein level (Fig. 5*B*), making it unlikely that the lack of cytokine production is merely due to the absence of cytosolic sensors.

Some miRNAs may be sorted into exosomes through association with nuclear ribonucleoproteins (RNP) (35). To search for potential EBER1 transport proteins, we analyzed the proteomics data for all known RNA-binding partners. We found few Pol III RNA-binding proteins enriched in LCL exosomes compared with control exosomes from EBV⁻ B cells (Fig. S5*A*). One of these, the major vault protein (MVP), associates with vRNA1-1 (36), which was enriched in LCL exosomes. However, we did not detect the lupus antigen (La/SSB) (Fig. 5*C*), the most commonly described binding-partner of EBERs. As expected, Hsp70, Alix, and β-Actin were detected in exosomes, whereas the nuclear protein EBNA1 was absent. To investigate whether EBER1 associates with cytoplasmic La before sorting into exosomes, we performed RNA immunoprecipitation experiments in the cytosolic fraction of latent EBV-infected LCLs. Strikingly, after confirming specific pull-down of the La protein with human La/SSB reactive sera, we detected full-length coimmunoprecipitated EBER1 as shown on gel (Fig. 5*D*). Moreover, when normalized to input with La/SSB nonreactive sera, we estimated a 1,000-fold enrichment of EBER1 with La/SSB reactive sera (Fig. 5*E*). In comparison, EBER2 pull-down was very low, consistent with the observation that EBER2 transcripts are relatively low in the cytosolic fraction (Fig. 5*E*) and not transferred via exosomes (Fig. S4*C*).

To investigate whether the association of EBER1 with cytoplasmic La is physiologically relevant in that it shields its 5′ppp motif from sensor detection, we reduced La protein level by small-interfering RNA (siRNA) knockdown and analyzed *IFNB1* expression. La-knockdown increased *IFNB1* transcription in LCLs, whereas MVP-knockdown had no such effect (Fig. 5*F* and Fig. S5 *B* and *C*). Thus, EBERs may evade detection by cytosolic

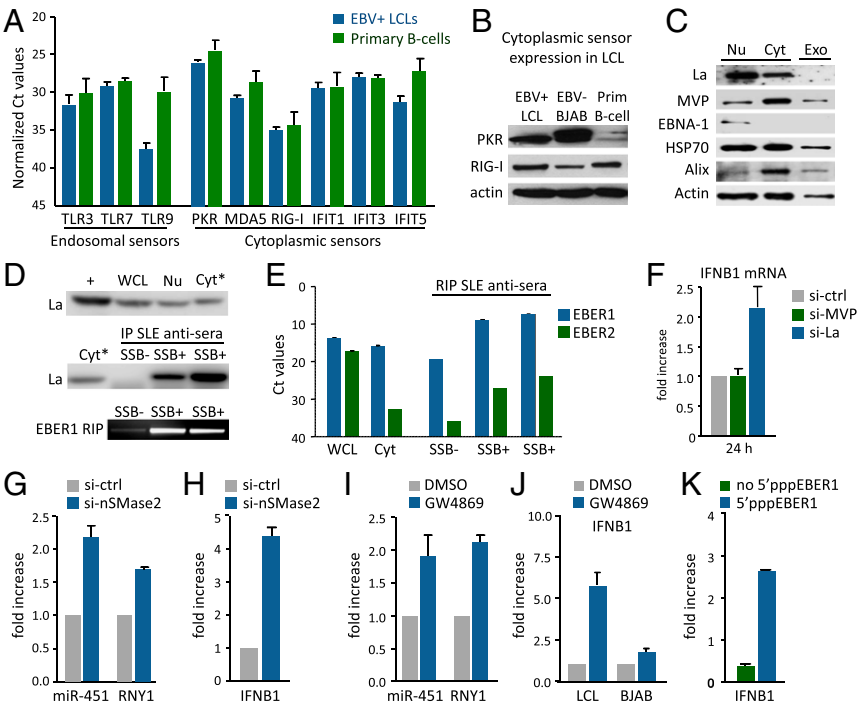

Fig. 5. EBER1 evades viral sensors by association with La. (*A*) qPCR analysis of viral sensors in EBV$^+$ LCLs (blue bars) and primary B cells (green bars). (*B*) Western blot analysis of PKR and RIG-I in EBV$^+$ LCLs, EBV$^-$ BJAB cells and primary B cells. (*C*) Western blot analysis of La, MVP, and indicated proteins in LCL nuclear (Nu), cytosolic (Cyt), and exosomal (Exo) fraction. (*D* and *E*) RNA immunoprecipitation (RIP) detects EBER1–La complexes in the cytosolic fraction. Western blot analysis of La in whole-cell lysate (WCL), nuclear and cytosolic fractions (*D*, *Top*). La immunoprecipitation in the cytosolic fraction using SSB$^-$ control antiserum and two different SSB$^+$ antisera (*Middle*). Detection of coimmunoprecipitated full-length EBER1 (*D*, *Bottom*). qPCR analysis of EBER1 and EBER2 in WCL, input (cytosol), and control (SSB$^-$) and La (SSB$^+$) immunoprecipitates (*E*). (*F*) siRNA knockdown experiments show that La- but not MVP-knockdown increases *IFNB1* mRNA expression in LCLs. (*G* and *H*) Intracellular levels of miR-451 and RNY1 (*G*) and *IFNB1* (*H*) in LCLs after treatment with siRNA against nSMAse2. (*I* and *J*) Intracellular levels of miR-451 and RNY1 (*I*) and *IFNB1* (*J*) in LCLs after treatment with GW4869. (*K*) *IFNB1* mRNA levels after LCL transfection with ivtEBER1 with or without 5′triphosphate.

sensors in latently infected B cells by interacting with the La protein, as previously suggested for respiratory syncytial virus-produced small RNAs (37). We also tested this in HekPRR reporter cells that expressed EBERs from nuclear plasmids. Despite sorting and trafficking into exosomes, we did not observe an increase in IFN-β promoter activation (Fig. S6 *A* and *B*). Because EBER1 is sorted into exosomes without La, we wondered whether interference of small RNA sorting at endosomes, might disrupt EBER evasion from sensors. To investigate this, we treated LCLs with a small-interfering RNA against nSMase2, a protein implicated in both exosome release and small RNA secretion (38). As expected, siRNA treatment elevated intracellular levels of miR451 and Pol III RNY1 (Fig. 5*G*). Importantly, this effect was associated with a significant increase in *IFNB1* transcription (Fig. 5*H*). Treatment of LCLs with GW4869, an inhibitor of nSMase2 function (39), yielded similar results (Fig. 5 *I* and *J*). Finally, transfection of 5′pppEBER1 directly into LCLs also increased *IFNB1* and ISG (*IFITM1* and *IFITM3*) transcription, confirming that endogenous sensors can sense 5′pppEBER1 molecules (Fig. 5*K* and Fig. S6 *C* and *D*). Thus, EBERs can be recognized in latently infected B cells, but interaction with La and sorting into exosomes prevent detection.

Exosomes from Latent EBV-Infected Cells Target Primary Tonsillar Plasmacytoid DCs. A prominent model of EBV persistence predicts that in healthy individuals newly infected naïve B cells follow a "normal" germinal center reaction in tonsillar tissue (40). Exosomes from infected B cells might transmit physiological messages to neighboring cells in these tissues. We cultured the primary tonsil cells in exosome-depleted medium and isolated the released tonsillar exosomes after 48-h culture. Interestingly, we could detect EBER1 transcripts in the exosomes of the tonsil cell population from EBV$^+$ donors (as confirmed by *EBNA1* Q-K DNA-PCR) (Fig. 6*A*).

To study exosome communication between EBV-infected B cells and surrounding cells, we first purified LCL exosomes by ultracentrifugation, yielding pure populations of vesicles ranging from 70 to 100 nm in size (Fig. 6*B*) and labeled these with PKH67, a green fluorescent dye. We incubated the PKH-exosomes with primary mononuclear tonsil cells from EBV$^-$ donors. We observed with confocal microscopy that only a small proportion of cells internalize the PKH-exosomes efficiently (Fig. 6*C*). FACS analysis revealed that tonsils harbor mainly B and T cells (up to 75% and 24%, respectively) and only minor populations of NK cells (CD3$^+$/CD56$^+$) and CD123$^+$/BDCA2$^+$ pDCs (Fig. 6*D* and Fig. S7*A*). Importantly, when incubating tonsil mononuclear cells with fluorescently labeled LCL or control BJAB exosomes, we found that CD123$^+$/BDCA2$^+$ pDCs internalize exosomes much more efficiently than the CD123$^-$/BDCA2$^-$ cell population (Fig. 6*E* and Fig. S7*B*). Moreover, assuming exosomes are added in excess, we observed that LCL exosomes are more efficiently internalized by pDCs compared with EBV$^-$ control exosomes. Finally, we found that EBV$^+$ LCL exosomes, but not control exosomes, induced *IFITM1* (but not *IFITM3*) transcription in tonsil mononuclear cells (Fig. 6*F*). This result argues against the possibility that PKH67 particles or PKH67-induced clustering of exosomes have a role in ISG induction. In vivo, IFITM proteins have various physiological functions and expression patterns, possibly explaining why we did not detect *IFITM3* induction in bulk tonsil cell populations (41, 42). As a clear sign of activation, CD40 surface expression was increased in pDCs upon interaction with unstained EBV$^+$ LCL exosomes (Fig. 6*G*).

As expected, tonsillar pDCs express endosomal RNA sensors *TLR7* and *TLR3*, more so than other tonsil cells, whereas *RIG-I*, *PKR*, and *MDA5* transcripts are not enriched (Fig. 6*H*). T-cell immunoglobulin mucin (TIM) proteins 1/4 exosome receptors on tonsillar pDCs are highly enriched at the RNA (Fig. 6*H*) and protein (surface) level as determined by FACS (Fig. 6*I*). We propose that EBER1-sensing in pDCs is mediated by exosome capture, possibly through surface TIM1 or -4 exosomes receptors (43).

EBER1 Transfer to Noninfected Cells in Vivo. To evaluate whether EBER1 molecules are taken up by pDCs in vivo, we studied pDC-infiltrated skin lesions of lupus erythematosus (LE) patients that suffer from chronically elevated EBV loads (44). We examined paraffin sections of skin biopsies by immunofluorescent staining. Using CD123 as a marker, we detected infiltration

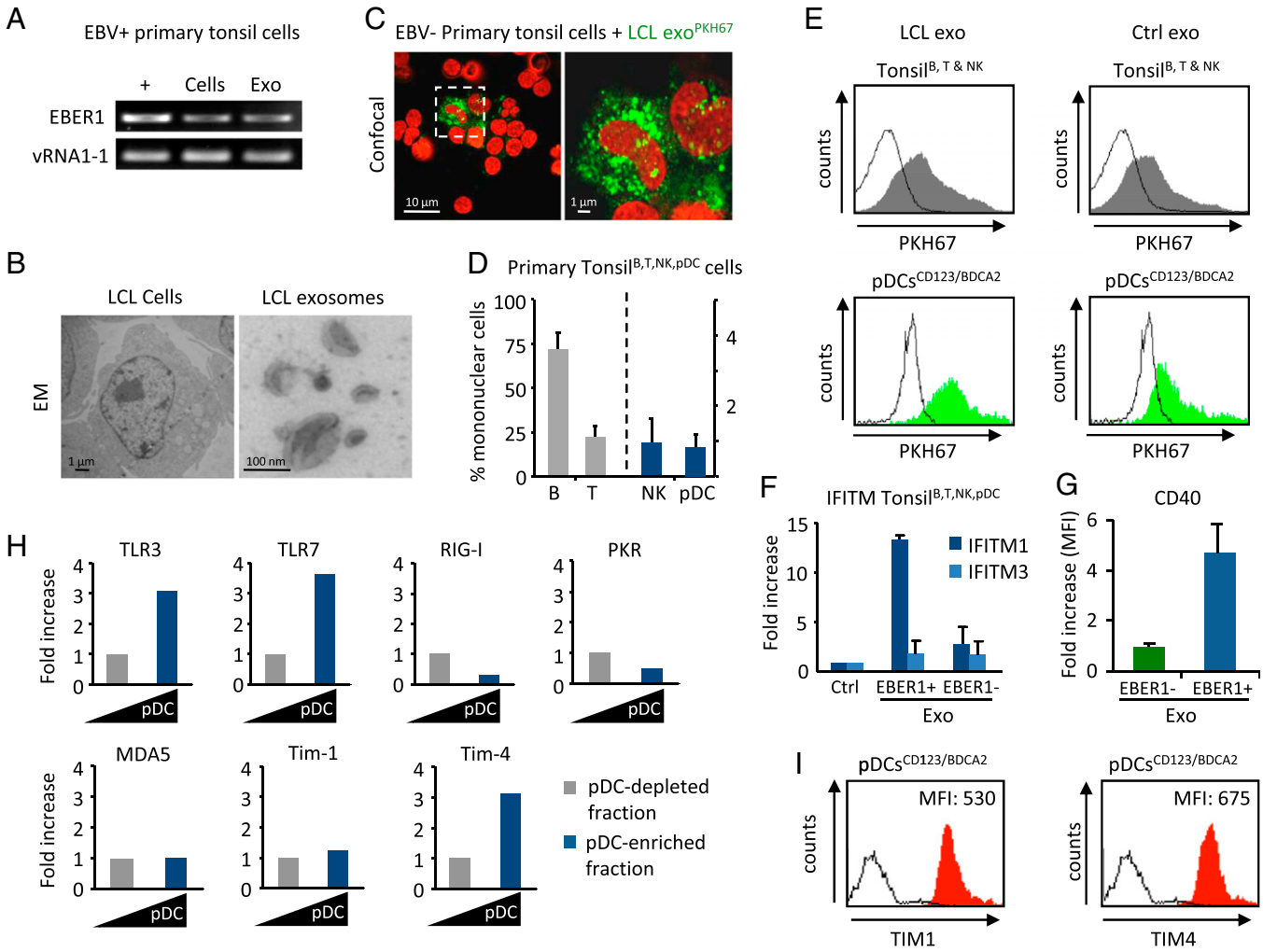

Fig. 6. Exosomes from latent EBV-infected cells are internalized by Tim1/4, TLR3/7-expressing pDCs. (*A*) Full-length EBER1 and vRNA1-1 in tonsil cells from EBV$^+$ donors and corresponding exosomes. (*B*) Electron microscopy micrograph of LCL cells and exosomes. (*C*) Confocal microscopy image of primary mononuclear tonsil cells (from EBV$^-$ donors) incubated with PKH67-labeled LCL exosomes. Nuclei are stained with TO-PRO-3. (*D*) Relative percentage of B, T, and NK cells and pDCs in the tonsil mononuclear cell population as analyzed by FACS (Fig. S7*A*). Graph shows the average percentage of cells from at least four different donors. (*E*) FACS analysis of tonsil B, T, and NK cells (*Upper*) and CD123$^+$/BDCA2$^+$ pDCs (*Lower*) incubated with PKH67-labeled LCL/BJAB exosomes. Tonsillar pDCs internalize LCL exosomes more efficiently compared with nonpDC. (*F* and *G*) *IFITM1* and *IFITM3* mRNA expression in tonsil cells (*F*) and CD40 (*G*) expression in the pDC subpopulation upon internalization of EBER1$^+$ or EBER1$^-$ exosomes. (*H* and *I*) Receptor expression in the pDC subpopulation compared with the pDC-depleted fraction by qPCR (*H*), and expression of TIM1 and TIM4 exosome receptors at pDC surface as analyzed by FACS (*I*). MFI, mean fluorescence intensity.

of pDCs in inflamed and LE tissues (Fig. 7 *A* and *B*). Immunofluorescent staining for CD40 revealed activation of the infiltrating pDCs in LE patient skin (Fig. 7*B* and Fig. S7*C*). Next, we extracted RNA from these tissues and performed EBER1 RT-PCR. In three of eight LE tissues, but not in healthy control skin (zero of four), we detected the presence of EBER1 molecules (Fig. 7*C*).

Because archived paraffin material can yield low intact RNA yield, we analyzed 10 additional frozen LE biopsies. In these tissues we measured very high levels of EBER1 transcripts, underscored by representing EBER1 levels as "infected LCL B-cell equivalents" (Fig. 7*D*). EBER1 levels in the LE tissues were significantly higher ($P = 0.033$) than in the control tissues, whereas EBV-DNA was virtually absent (Fig. 7 *D* and *E*), suggesting that EBER1 signals are not derived from actual EBV-infected cells. In accordance with this conclusion, no EBER1 nuclear staining was detected on systemic lupus erythematosus (SLE) skin sections by in situ EBER hybridization (Fig. S7*D*). In addition, we found elevated EBV-BART miRNAs in the inflamed skin tissues, the expression levels of which positively correlated with that of EBER1, whereas no correlation was observed between hsa-miR21 and EBER1. In contrast, BHRF1-2 miRNAs expressed during EBV-lytic stage or in latency type III LCLs (45) were not detected, suggesting that the EBV transcripts in these lesions were derived from latently infected cells (Fig. 7 *E*–*I*).

Discussion

Latent EBV-infected cells continuously produce small noncoding EBERs that can be detected by PRRs and ignite proinflammatory responses (9, 46, 47). It remained unclear whether EBERs gain access to cytoplasmic compartments to instigate antiviral immunity (21). Here we show that cytoplasmic 5′pppEBER1 is selectively sorted into exosomes that are internalized by pDCs, triggering antiviral immunity. The exosomal messenger pathway of cellular stress and infection may be an evolutionarily conserved protective mechanism that may cause disease if not properly regulated.

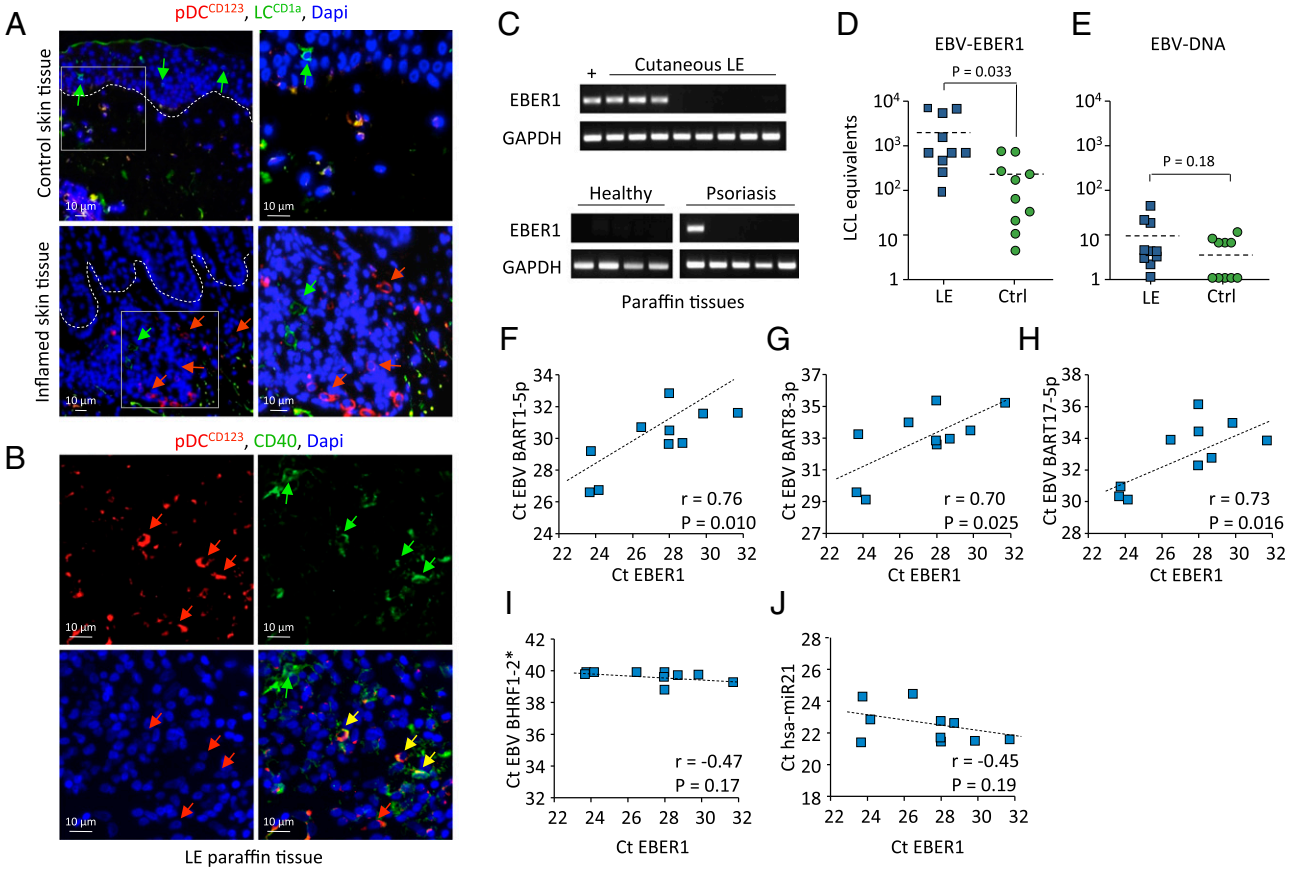

Fig. 7. EBER1 is present in pDC-infiltrated SLE skin lesion in the absence of EBV-DNA. (*A*) Immunofluorescence staining of paraffin-embedded inflamed and healthy skin tissue. CD123 is used as pDC marker and CD1a as Langerhans cell (LC) marker. Nuclei are stained with DAPI. (*B*) Immunofluorescence staining of cutaneous LE paraffin tissues shows CD123^{+}/CD40^{+} cells. (*C*) qPCR of EBER1 in lupus erythematosus and control (healthy and psoriasis) tissues. (*D* and *E*) qPCR analysis of EBER1 (*D*) and EBV-DNA (*E*) in control and cutaneous LE frozen tissues. qPCR data are expressed as EBV^{+} LCL equivalents. (*F–J*) Correlation of EBV-miRNAs and hsa-miR-21 with EBER1 in skin tissues analyzed by qPCR.

EBERs can bind to viral (EBNA1) (48) and host RNA binding proteins (49). EBER1 and EBER2 associate with La, an abundant RNP in the nucleus of latently infected B cells (50) that binds nascent Pol III transcripts, protecting 3′ ends from exonucleases (51). The abundance of small RNAs in EBV-infected cells is linked to EBNA1 protein expression and the transcription factor TFIIB, which raises Pol III activity (32). Whereas EBER1 interacts with the La phosphoprotein in nuclei, our data show that a fraction of EBERs interact strongly with cytoplasmic La. Although the exact physiological function of this interaction is unclear, knock-down of La protein led to increased *IFNB1* transcription (Fig. 5*F*), similar to what has been observed in respiratory syncytial virus-infected cells. Here, La associates with leader small RNAs (leRNA) and knock-down caused *IFNB1* transcription in infected cells (37). EBERs do not acquire a 5′-triphosphate cap-structure, making them susceptible for detection by cytosolic sensors. Indeed, introducing "naked" 5′pppEBER1 directly into RIG-I–expressing latent-infected B cells triggers *IFNB1* transcription (Fig. 5*K*), indicating that the endogenous pool of cytoplasmic 5′pppEBERs fails to activate cytosolic sensors.

Prior studies showed that EBV-EBER transcripts produced from cytoplasmic AT-rich (EBV)DNA triggers RIG-I signaling (52, 53). Our studies imply that Pol III viral transcripts transferred through exosomes and not herpes virus DNA are detected by immunological guardian cells during persistence. It was previously shown that EBER1 complexed to RNPs is detected by TLR3, suggesting that double-stranded RNA loops act as PAMPs. This finding is distinct from exosome-delivered EBER1, where enzymatic removal of 5′ppp rendered EBER1 molecules noninflammatory (Fig. 4 *E–H*). Thus, 5′ppp-recognizing sensors (i.e., RIG-I, IFIT, or PKR) are more likely to have a role in the recognition of the exosome-delivered small RNA. Moreover, only EBER1 exosomes induced *IFIT* (interferon induced proteins with tetratricopeptide repeats) 1 and 3 RNA sensor mRNA expression in recipient DCs (Fig. S6*E*). This finding suggests that 5′pppRNA exosomes transport stress-related signals to neighboring cells, priming them for 5′ppp-RNA detection. Interestingly, breast cancer stroma cells under stress deliver endogenous 5′pppRNAs that are recognized by RIG-I in tumor cells (54). Thus, a conserved intercellular pathway exists in which stress signals in the form of small RNAs are transported between cells via exosomes.

Herpes simplex virus-1 DNA has been detected in the cytosol of infected cells, but its origin (i.e., nuclear, cytoplasmic, or endosomal) remained unclear (55). Upon viral entry, EBV-DNA in the cytosol may be transcribed by RNA pol III, producing cytoplasmic EBERs activating RIG-I (52, 53); although unlikely, we cannot exclude the possibility that some EBV-DNA accesses the cytosol of latently infected LCLs. We propose that by interaction with La and sorting into exosomes, nuclear 5′pppEBER1 eludes cytosolic detection in established latent-infected cells. In contrast, EBERs produced from incoming (cytosolic) EBV-DNA by Pol III, may be sensed by RIG-I in newly EBV-infected B cells. Possibly, resting primary B cells have lower amounts of cytosolic La protein, making them particularly prone to EBER sensing. Although EBER–La complexes may be released in a

vesicle-independent manner, we did not detect La in exosomes by proteomics or by Western blotting (9). We used sucrose density-based purification of exosomes, which may explain why we do not detect La in exosomes preparations (23). In addition, in EBV-infected LCLs, but not in EBV$^-$ control cells, inhibiting small RNA sorting raises *IFNB1* transcription (Fig. 5 *G–J*). Furthermore, EBER1, but not EBER2, was sorted and released via exosomes, consistent with EBER2 having a nuclear function (56).

Constitutive expression of TLRs and a predisposition for rapid and massive type I IFN release by pDCs pose a risk for developing autoimmunity (57). Exosomes from EBV-infected B cells could trigger inflammation directly or indirectly by elevating the expression of 5′pppRNA-sensors (IFIT proteins) (58) that can recognize self-RNA (Fig. S6*E*). Although a role for extracellular viral small RNAs has not been considered, pDC infiltration in skin lesions of autoimmune patients is frequently observed (57, 59, 60). There is evidence that the EBV-related herpes simplex virus is recognized by innate sensors during latency in skin-infiltrated pDCs (61, 62). Moreover skin inflammation can be a consequence of UV-stress, because damaged nuclear small RNAs from keratinocytes trigger TLR3, presumably in a non–cell-autonomous fashion (18). Because pDCs constitutively express TLR3, they are a likely candidate cell-type involved in tissue inflammation. In addition, antiviral immunity triggered by exosomal transfer of viral RNA to pDCs has been suggested in HCV infection (3). HCV persistence and pathology, depends on strategies evolved to avoid innate recognition in infected cells (2). It will become important to resolve how pathogenic (viral) RNAs are incorporated and transported via exosomes, as the packaging machineries of virion capsids are presumably lacking. Recently, we and others showed that B cells latently infected with EBV export functional miRNAs to uninfected cells via exosomes (25, 58).

One question that still needs addressing is whether bystander recognition of latent EBV-infected cells contributes to life-long persistence. Recent studies in humanized mice suggest that EBV-deficient in EBER1 is not impaired in establishing persistence (8), whereas EBER2 increases LMP2 transcription (56). One could imagine that EBER1-activated pDCs during primary EBV infection primes NK cells to restrict outgrowth (63). Indeed, failure to respond to primary EBV infection can be fatal in predisposed individuals suffering from X-linked lymphoproliferative syndrome (64). During persistence, EBER recognition may keep the pool of latent-infected memory B cells that do not overtly expose EBV-antigens, at low levels. The absence of detectable BHRF1 miRNAs and EBV-genomes, but the positive correlation between EBER1 transcripts and EBV-BART miRNAs in exosomes, strongly suggest a joint delivery mechanism from latently infected cells (45). Our findings in vitro were made with naturally infected latency III LCLs that grow rapidly and release well-defined exosomes (65), whereas the most dominant infected cell type in lymphoid tissues and circulation are presumably memory B cells (40). Latency I memory B cells have sustained expression of EBV-noncoding RNAs, except the BHRF1-miRNAs (45). Although resting, these cells may retain the ability to release exosomes that have incorporated EBER1. However, latency III cells have been detected in circulation of individuals under immune suppression (44), and may represent an additional source of EBER1$^+$ exosomes. Although chronic sensing of latent EBV may lead to disease in predisposed individuals, EBER1 recognition during early stages of EBV infection is possibly protective for the host, as at this time adaptive immune responses are not yet functional.

In conclusion, we propose that EBER1-containing exosomes have a role in immune recognition of latently EBV-infected B cells, which is a normal feature of EBV persistence, but maybe exacerbated in EBV-linked inflammatory autoimmune diseases like lupus erythematosus and multiple sclerosis.

Methods

Exosome Isolation and Purification. Exosomes were isolated by differential centrifugation, as previously described (30). When indicated, exosomal RNA was treated with CIP (New England Biolabs) to remove 5′-triphosphate. For uptake experiments, purified exosomes were labeled with PKH67 dye (Sigma-Aldrich), as described previously (25).

Microarray Analysis. DC RNA quantity and quality were determined on a 2100 Bioanalyzer, and samples were subjected to Agilent high-density Microarrays according to the manufacturer's protocol. Data were truncated for maximum intensities at 10,000 and filtered for minimum intensities (45). Fold-change was set higher than 1.5 at least in one sample per group. Quantile normalization was applied. The resulting 10.383 filtered genes were subjected to unpaired class comparison univariate test to select differentially expressed genes ($\alpha = 0.0005$). Log_2 ratio between the geometrical mean of intensities for each class were calculated. Genes with fold-change greater than 2 were selected. Analyses were performed using BRB-ArrayTools, developed by Richard Simon and the BRB-ArrayTools Development Team (linus.nci.nih.gov/BRB-ArrayTools.html).

Stimulation of DCs and Coculture. The 5′pppEBER1 (100 ng) was added to or transfected in immature DCs using Lipofectamine 2000 (Life Technologies) for 24 h. Coculture experiments were performed as previously described (25).

All clinical samples and primary cell culture (i.e., dendritic cells and tonsil cells) were obtained with informed consent, evaluated by the Vrije Universiteit Medical Center ethical board committee and used in compliance with the Declaration of Helsinki.

Additional methods are included in *SI Methods*. See Table S1 for adapter, primers, and probes.

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