Epstein–Barr virus exploits host endocytic machinery for cell-to-cell viral transmission rather than a virological synapse

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Epstein–Barr virus (EBV) establishes a lifelong latent infection in B lymphocytes and often is found in epithelial cells. Several lines of evidence indicate that viral transmission mediated by cell-to-cell contact is the dominant mode of infection by EBV for epithelial cells. However, its detailed molecular mechanism has not been fully elucidated. We investigated the role of host membrane trafficking machinery in this process. We have found that adhesion molecules critical for this process are expressed in EBV-positive and -negative Burkitt’s lymphoma (BL) cells and multiple epithelial cell lines. Treatment with blocking antibodies against β1 and β2 integrin families and their ligands suppressed EBV transmission in a dose-dependent manner. We also confirmed that adhesion molecules are upregulated in co-cultured BL cells. Immunofluorescence staining revealed that the intracellular adhesion molecule 1 (ICAM-1) distributed to the cell surface and partially co-localized with recycling endosomes in co-cultured BL cells. Moreover, cell-to-cell EBV transmission was inhibited upon blocking endocytic recycling by expression of a dominant-negative form of a small GTPase Rab11 or by knockdown of Rab11, supporting the notion that the endocytic pathway-dependent trafficking of ICAM-1 to the cell surface of BL cells contributes to viral transmission by stabilizing cell-to-cell contact between the donor cells and recipient cells. Finally, we demonstrated that co-cultivation upregulated clathrin-mediated endocytosis in the recipient cells, allowing EBV to be internalized. Taken together, our findings demonstrate that EBV exploits host endocytic machinery in both donor and recipient cells, a process which is facilitated by cell-to-cell contact, thereby promoting successful viral transmission.

INTRODUCTION

Epstein–Barr virus (EBV), a human gamma herpesvirus, establishes a persistent latent infection in B lymphocytes and often is found in epithelial cells. EBV also causes lymphomas and epithelial malignancies such as Burkitt’s lymphoma (BL), Hodgkin’s disease, nasopharyngeal carcinoma and gastric cancer (Kieff & Rickinson, 2001).

The mechanism by which EBV infects B lymphocytes has been well characterized. EBV attaches to B lymphocytes through a direct interaction of the EBV major outer envelope glycoprotein complex gp350/220 with the complement receptor CD21 (Fingeroth et al., 1999; Nemerow et al., 1987; Tanner et al., 1987, 1988), leading to endocytosis-mediated internalization of EBV. Fusion of the viral envelope with the endosomal membrane of B cells is triggered by the interaction of a second envelope glycoprotein, gp42, with HLA class II (Li et al., 1997; Iizasa et al., 2012; Nemerow et al., 1987; Oda et al., 2000). In contrast, the mechanism by which EBV infects epithelial cells remains less well defined. Infection of cell-free EBV into epithelial cells appears to be mediated by fusion of the viral envelope with the cellular plasma membrane. Human epithelial cells are CD21-negative or some epithelial cells in culture express CD21 at low levels, resulting in a high resistance to cell-free EBV infection (Fingeroth et al., 1999; Imai et al., 1998). Several lines of evidence indicate that cell-to-cell viral transmission is a dominant mode for infection of EBV into epithelial cells. The efficiency of EBV infection in epithelial cells is significantly enhanced by co-culturing them with
EBV-positive B cells relative to cell-free infection (Chang et al., 1999; Imai et al., 1998; Nanbo et al., 2012; Speck & Longnecker, 2000). These studies support a model in which EBV-infected B cells that migrate into the epithelial stroma or intraepithelial space contribute to efficient EBV transmission into epithelial cells via cell-to-cell contact. However, the detailed molecular mechanisms of cell-to-cell EBV transmission remain to be fully elucidated.

It has been shown that cell-to-cell infection of retroviruses such as human T-cell lymphotropic virus (HTLV) and human immunodeficiency virus (HIV) is mediated by the virological synapse (VS). The VS is an actin- and microtubule-dependent stable adhesive junction across which retroviruses can be efficiently transferred from virus-infected dendritic cells or T cells to non-infected target T cells without cell–cell fusion (Agosto et al., 2015; Jolly & Sattentau, 2004). In the process of the VS formation, cell organelles such as the microtubule-organizing centre (MTOC) and endosomes are reoriented toward the interface of contacted cells, resulting in active recruitment of viral antigens, cellular receptors and adhesion molecules toward the site of conjugation (Jolly & Sattentau, 2004; Zhong et al., 2013). Assembly and budding of retroviruses is targeted to the VS, leading to subsequent fusion of the viral envelope with the plasma membrane or with the endosome of recipient cells (Bosch et al., 2008; Dale et al., 2011; Puigdomènech et al., 2009).

Previously we established an assay to assess the efficiency of viral transmission mediated by cell-to-cell contact by co-culturing latently EBV-infected cells and EBV-negative epithelial cells. By use of this assay, we demonstrated that cell-to-cell contact induces multiple cell signalling pathways in BL cells and epithelial cells, contributing to the induction of the viral lytic cycle in BL cells and the enhancement of viral transmission to epithelial cells (Nanbo et al., 2012).

In the present study, we have assessed the role of the host membrane trafficking machinery in cell-to-cell EBV transmission. We observed that critical adhesion molecules are upregulated in EBV-positive and -negative BL cells when co-cultured with epithelial cells. In particular, co-cultivation induced the trafficking of intracellular adhesion molecule 1 (ICAM-1) to the cell surface in BL cells in a vesicle recycling-dependent manner. We also observed that direct cell-to-cell contact enhanced clathrin-dependent endocytosis in recipient cells, which supported progeny EBV virions entering the recipient cells. There were no significant differences between EBV-positive and -negative BL cells in the expression levels of adhesion molecules in co-cultured cells, the efficiency of cell–cell contact-mediated upregulation of adhesion molecules, the recycling, endosome-dependent translocation of ICAM-1 and the upregulation of endocytosis. Taken together, our observations show that EBV exploits pre-existing host endocytic machinery for its establishment of efficient viral transmission, a mechanism distinct from that of the VS employed by HIV.

**RESULTS**

**Identifying adhesion molecules expressed on Akata cells and epithelial cells**

Previously we established an assay to assess the efficiency of cell-to-cell transmission of EBV by co-culturing EBV-positive BL cells with EBV-negative epithelial cells (Nanbo et al., 2012). We used the BL-derived Akata-EBV-eGFP cells, which are latently infected with a recombinant Akata-derived strain of EBV encoding eGFP (Maruo et al., 2001a), with the EBV-negative human gastric epithelial cell lines (AGS and NU-GC-3 cells) and African green monkey kidney epithelial cell line (Vero-E6 cells). The transmission of EBV-eGFP into the epithelial cells was analysed by quantifying the percentage of eGFP-positive cells with flow cytometry.

The role of adhesion molecules, such as ICAM-1 and leukocyte function-associated antigen 1 (LFA-1), in the VS-mediated retrovirus infection has been characterized and found to be critical (Jolly & Sattentau, 2004; Zhong et al., 2013).

In the present study, we investigated the role of adhesion molecules in cell-to-cell transmission of EBV. First we assessed the expression of CD11a (or αL subunit) and CD18 (or β2 subunit), which are components of αLβ2 integrin (or LFA-1) and known to participate in VS-mediated viral transmission, in EBV-positive Akata (Akata+) and -negative Akata (Akata−), Vero-E6 cells, AGS cells and human nasopharyngeal carcinoma CNE1 cells by flow cytometry. We also examined the expression of CD49d (or α4 integrin subunit), CD49e (or α5 subunit) and CD29 (or β1 subunit), which are components of α4β1 (or very late antigen 4; VLA4) and α5β1 integrins (or VLA5) in these cell lines. Moreover, the expression of vascular cell adhesion molecule 1 (VCAM-1) and fibronectin, which are ligands for β1 integrin, and ICAM-1, which is a ligand of β2 integrin, were analysed. We also assessed the expression of αvβ5 and αvβ6, two molecules which have been shown to interact with gHgL and lead to the fusion of viral envelope and the plasma membrane of epithelial cells (Chesnokova & Hutt-Fletcher, 2011). Akata+ (Fig. 1a), Akata− (Fig. 1b), Vero-E6 (Fig. 1c), AGS (Fig. 1d) and CNE1 (Fig. 1e) cells expressed all of these adhesion molecules. Although the expression levels of individual adhesion molecules varied among the examined cell lines, no significant differences were observed between EBV-positive and -negative BL cells.

**Blocking antibodies to adhesion molecules inhibit cell-to-cell transmission of EBV**

We examined the role of these adhesion molecules and their ligands in cell-to-cell transmission of EBV with blocking antibodies. Akata− EBV-eGFP cells and individual epithelial cells were pretreated with blocking antibodies against CD49d, CD49e, CD29, VCAM-1, ICAM-1, αvβ6 or αvβ6 followed by co-cultivation in the presence of each of these antibodies. To enhance our detection of viral transmission, we cross-linked the cell surface IgG of Akata− EBV-eGFP by adding F(ab’)2 fragments of goat anti-human IgG.
polyclonal antibody (αhIgG) to induce the viral lytic cycle (Takada, 1984; Takada et al., 1991; Takada & Ono, 1989).

Consistent with our previous report (Nanbo et al., 2012), co-culturing with Akata− EBV-eGFP led to approximately

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**Fig. 1.** Expression of adhesion molecules in Akata cells and epithelial cells. Akata+ cells (a), Akata− cells (b), Vero-E6 cells (c), AGS cells (d) or CNE1 cells (e) were incubated with antibodies for CD49d, CD49e, CD29, CD11a, CD18, VCAM-1, fibronectin, ICAM-1, αvβ5, αvβ6 or oestrogen receptor (ER). The expression of individual molecules was revealed with Alexa Fluor 488-labelled secondary antibodies (boldface lines) by flow cytometry. As a control, cells were incubated with the secondary antibody (thin lines). The experiments were performed three times independently and representative histograms are presented ER.
Fig. 2. Effect of neutralizing antibodies on cell-to-cell EBV transmission. (a) EBV-eGFP is transmitted to various epithelial cells. Vero-E6, AGS or CNE1 cells were co-cultured with Akata<sup>−</sup> EBV-eGFP cells in the absence or presence of αhlG for 24 h to induce EBV’s productive cycle. The percentages of eGFP-positive, infected epithelial cells were analysed by flow cytometry. The experiment was performed three times independently, and the mean values and their SD are shown for each condition. (b) Effect of blocking antibodies on cell-to-cell contact-mediated EBV transmission into Vero-E6 cells. Akata<sup>−</sup> EBV-
eGFP cells were treated with (grey bars) or without (white bars) 1% αIgG for 2 h at 37 °C and washed with medium. Akata − EBV-eGFP cells or Vero-E6 cells were pretreated with various amounts of blocking antibodies for CD49d, CD49e, CD29, VCAM-1, ICAM-1, αβ5, αβ6 or ER (0.1, 0.2, 0.5, 1.0 and 10 μg ml⁻¹) for 30 min at 37 °C. Pretreated cells were co-cultured for 24 h in the presence of individual antibodies. The percentages of EBV-eGFP positively infected Vero-E6 cells were analysed by flow cytometry. The infection efficiency is shown relative to the value when co-cultured with αIgG-untreated Akata − EBV-eGFP cells in the absence of antibodies. The experiment was performed three times independently and the mean and its SD are shown in each condition. *P<0.05 versus respective control. **P<0.01 versus respective control (Student’s t-test).

5% of the cell population being infected. αIgG treatment increased the detected transmission efficiency up to approximately 11% in Vero-E6 cells (Fig. 2a). Approximately 5 and 7% of the cell population was infected by EBV-eGFP in the presence of αIgG in AGS and CNE1 cells, respectively (Fig. 2a).

All antibodies against β1 and β2 integrins and their ligands suppressed EBV transmission to Vero-E6 (Fig. 2b), AGS and CNE1 (Fig. 2c) cells in a dose-dependent manner. In contrast, treatment with the antibodies against αβ5, αβ6 and oestrogen receptor (ER), which are also expressed in both Akata cells and epithelial cells (Fig. 1), exhibited no effect on viral transmission (Fig. 2b, c), suggesting that cell-to-cell transmission of EBV was specifically dependent on cell contact through an interaction between β1 and β2 integrins and their ligands. Moreover, our findings also indicate that CD49e, VCAM-1 and fibronectin, which are not known to participate in VS-mediated retrovirus infection, do contribute to the cell-to-cell transmission of EBV.

Cell-to-cell contact upregulates expression of adhesion molecules in co-cultured BL cells

We assessed whether direct cell-to-cell contact modulates the expression of β1 and β2 integrins and their ligands in co-cultured cells to determine if co-cultivation facilitates viral transmission beyond the juxtaposing of cells. Akata + or Akata − cells were co-cultured with Vero-E6 cells for 6 h and harvested separately. To ensure the harvested cells did not contain co-cultured donor cells, the expression of HLA-DR or caveolin-1 (Cav1) in the harvested cells was analysed by flow cytometry. HLA-DR is the class II histocompatibility molecule constitutively expressed on antigen-presenting cells including B cells, and T lymphocytes only after activation (Trowsdale et al., 1991). Cav1 is an epithelial cell marker (Couet et al., 2001). Both Akata + and Akata − cells expressed HLA-DR similarly, and its expression was upregulated after a 6 h long co-cultivation (Fig. 3a, c). The expression of HLA-DR in Vero-E6 cells was low compared with that in BL cells and its expression did not change significantly (Fig. 3a, c).

Cav1 was expressed in Vero-E6 cells similarly in the presence and absence of cell contact (Fig. 3b, d). EBV-positive and -negative Akata cells were almost Cav1-negative and its expression did not change by cell contact (Fig. 3b, d). The data indicate that the BL cells and Vero-E6 cells were successfully harvested separately. CD29, VCAM-1 and ICAM-1 were found to be upregulated in both Akata + and Akata − cells after a 6 h long co-cultivation (Fig. 3e, g). These BL cells expressed proteins at different levels; CD49d and ICAM-1 were higher in Akata +, and CD11a and fibronectin were higher in Akata − (Fig. 3e, g). In contrast, the recipient Vero-E6 cells did not change their levels of individual adhesion molecules when co-cultivated with either the EBV-positive or -negative BL cells (Fig. 3f, h). These measurements show that critical adhesion molecules are upregulated in BL cells on their contact with epithelial cells.

Cell-to-cell contact promotes the translocation of ICAM-1 to the plasma membrane of co-cultured BL cells

Because critical adhesion molecules were upregulated in co-cultured EBV-positive and -negative BL cells (Fig. 3e, g), we examined the kinetics of expression of ICAM-1 to determine if its increased expression could contribute to transmission of EBV. ICAM-1 was upregulated in both EBV-positive and -negative Akata cells after only 1 h long co-cultivation, and its upregulated status was maintained for 24 h (Fig. 4a, c). In contrast, the expression of ICAM-1 did not change and was even slightly downregulated in co-cultured Vero-E6 cells (Fig. 4b, d). We also examined the subcellular distribution of ICAM-1 in co-cultured cells by immunofluorescent staining. In the absence of cell-to-cell contact, ICAM-1 was distributed in the plasma membrane and cytoplasm as punctate signals in EBV-positive and -negative Akata cells (Fig. 4e, f). ICAM-1 moved to the plasma membrane after a 2 h long co-cultivation, and the distribution persisted up to 24 h, positioning it specifically and temporally to participate in EBV’s transmission. In contrast, ICAM-1 was predominantly distributed diffusely in the cytoplasm in Vero-E6 cells and its distribution did not change under co-cultivation (Fig. 4g, h).
Fig. 3. Effect of cell-to-cell contact on expression of adhesion molecules in co-cultured cells. (a, b) Expression of HLA-DR and Cav1 in co-cultured cells. Akata⁺ cells and Akata⁻ cells were co-cultured with (unbroken line) or without (dashed line) Vero-E6 cells for 24 h and expression of HLA-DR (a) or Cav1 (b) in Akata⁺ cells (top, left) and Akata⁻ cells (top, right) was analysed by flow cytometry. Expression of HLA-DR (a) or Cav1 (b) in Vero-E6 cells co-cultured with (unbroken line) or without (dashed line) Akata⁺ cells (bottom, left) or Akata⁻ cells (bottom, right) was analysed by flow cytometry. As a control, cells were incubated with secondary antibodies (shaded peak). Representative peak are shown. (c) Expression of HLA-DR in co-cultured cells. Akata⁺ or Akata⁻ cells were co-cultured with Vero-E6 cells for 24 h. Expression of HLA-DR in Akata⁺ cells (white bars) and Akata⁻ cells (grey bars) was analysed by flow cytometry (left). Expression of HLA-DR in Vero-E6 cells co-cultured with Akata⁺ cells (white bars) and Akata⁻ cells (grey bars) was analysed by flow cytometry (right). The data are normalized to the expression of HLA-DR in Akata cells without cell contact. The experiment was performed three times independently, and the mean values and their SD are shown for each condition. **P<0.01 versus respective control. NS, Not significant (Student’s t-test). (d) Expression of Cav1 in co-cultured cells. Akata⁺ or Akata⁻ cells were co-cultured with Vero-E6 cells for 24 h. Expression of Cav1 in Akata⁺ cells (white bars) and Akata⁻ cells (grey bars) was analysed by flow cytometry (left). Expression of Cav1 in Vero-E6 cells co-cultured with Akata⁺ cells (white bars) and Akata⁻ cells (grey bars) was analysed by flow cytometry (right). The data are normalized to the expression of Cav1 in Vero-E6 cells without cell contact. (e, f). Effect of cell–cell contact on expression of adhesion molecule in co-cultured cells. Akata⁺ cells and Akata⁻ cells were co-cultured with (unbroken line) or without (dashed line) Vero-E6 cells for 6 h. Expression of individual molecules was analysed by flow cytometry in Akata⁺ cells (left) and Akata⁻ cells (right) (e), and Vero-E6 cells co-cultured with Akata⁺ cells (left) or Akata⁻ cells (right) (f). As a control, cells were incubated with secondary antibodies (shaded peak). Representative peak are shown. (g, h) Effect of cell–cell contact on expression of adhesion molecule in co-cultured cells. Akata⁺ cells and Akata⁻ cells were co-cultured with Vero-E6 cells for 6 h. Expression of individual molecules was analysed by flow cytometry in Akata⁺ cells (white bars) and Akata⁻ cells (grey bars) (g), and Vero-E6 cells co-cultured with Akata⁺ cells (white bars) or Akata⁻ cells (grey bars) (h). The data are normalized to the expression of individual molecules in the cells without cell contact. The experiment was performed three times independently and the mean values and their SD are shown for each condition. *P<0.05 versus respective control. **P<0.01 versus respective control (Student’s t-test).
Some ICAM-1 localized to recycling endosomes in co-cultured BL cells

We investigated the mechanism by which ICAM-1 distributed to the plasma membrane in co-cultured BL cells (Fig. 4e, f). Previous studies have demonstrated that endocytic recycling contributes to the transport of various adhesion molecules to the plasma membrane (Desclozeaux et al., 2008; Diestel et al., 2007; Lock & Stow, 2005; Mamdouh et al., 2003). To determine whether this pathway is also involved in the cell-to-cell contact-induced translocation of ICAM-1, we investigated the subcellular localization of Rab11, a small GTPase in co-cultured Akata cells, by immunofluorescent staining. Rab11 associates with recycling endosomes and regulates exocytosis and recycling processes to transport proteins to the cell surface (Kelly et al., 2012; Ren et al., 1998; Welz et al., 2014). Rab11 also localizes to the trans-Golgi network and post-Golgi vesicles, and has been implicated in the trafficking between the trans-Golgi network and the endosomal recycling compartments through the regulated secretion pathway (Gromov et al., 1998). In the absence of co-cultivation, ICAM-1 (green) distributed in the cytoplasm and plasma membrane as punctate signals in EBV-positive and -negative Akata cells (Fig. 5a, b, top, left). Rab11 (red) predominantly distributed in the perinuclear regions and some fraction of the protein distributed in the cytoplasm and the plasma membrane (Fig. 5a, b, top, middle), which did not co-localize with ICAM-1. In contrast, in co-cultured EBV-positive and -negative Akata cells, ICAM-1 (green) distributed to the periphery of the cells and its cytoplasmic fraction partially co-localized with Rab11 (Fig. 5a, b, bottom), consistent with ICAM-1 being translocated to the plasma membrane via
Fig. 4. Effect of cell-to-cell contact on distribution of ICAM-1 in co-cultured cells. (a, b) Kinetics of ICAM-1 expression in co-cultured cells. Akata+ cells or Akata− cells were co-cultured with Vero-E6 cells for various times. The expression of ICAM-1 was analysed by flow cytometry in Akata+ cells (left) and Akata− cells (right) (a) and Vero-E6 cells were co-cultured with Akata+ cells (left) and Akata− cells (right) (b). The data are normalized to the expression of ICAM-1 in the cells without cell contact. As a control, cells were incubated with secondary antibodies (shaded peak). Representative peak are
Rab11-dependent recycling. We also visualized the distribution of ICAM-1 and Rab11 in a series of Z-stacks in co-cultured Akata$^+$ cells. ICAM-1 diffusely distributed to the peripheral region of the plasma membrane (Fig. 5c), which was distinct from the tight distribution of adhesion molecules that is observed in the VS.

![Fig. 5](http://jgv.microbiologyresearch.org) **Fig. 5.** Distribution of ICAM-1 and of recycling endosomes in co-cultured Akata cells. (a, b) Distribution of Rab11 in co-cultured Akata cells. Akata$^+$ cells and Akata$^-$ cells were co-cultured with Vero-E6 cells for 1 h. The distributions of ICAM-1 (left, green) and of Rab11 (middle, red) in Akata$^+$ (a) and Akata$^-$ cells (b) were examined by immunofluorescent staining (bottom). As a control, the distributions of ICAM-1 and of Rab11 in the cells without cell contact were analysed (top). The nuclei were counterstained with DAPI. Bars, 10 µm. (c) Z-stack images of distribution of ICAM-1 and Rab11 in co-cultured cells were determined by immunofluorescent staining in a series of Z-stack images that were collected at 0.7 µm intervals. The nucleus was counterstained with DAPI. Bar, 10 µm.
**Rab11-dependent recycling endocytic pathway contributes to the EBV transmission mediated by cell-to-cell contact**

Because ICAM-1 partially co-localized with Rab11 in the cytoplasm of co-cultured BL cells (Fig. 5), we investigated the role of Rab11 in cell-to-cell contact-mediated EBV transmission. We transiently expressed a dominant-negative form of Rab11 that has an amino acid substitution (N25S) (Chen et al., 1998) in Akata− EBV-eGFP cells (Fig. 6a) and assessed its effect both on the distribution of ICAM-1 in donor cells and on EBV transmission. Expression of Rab11 N25S in Akata− EBV-eGFP cells suppressed translocation of ICAM-1 to the plasma membrane of donor cells (Fig. 6b, c) and viral transmission (Fig. 6d). We also knocked down Rab11a and Rab11b isoforms in Akata− EBV-eGFP cells with a small interfering+ RNA (siRNA). Rab11a and Rab11b share 90 % amino acid sequence identity. Rab11a is expressed ubiquitously, predominantly localizes to recycling endosomes and functions in the recycling of a wide range of molecules to the cell surface (Gromov et al., 1998). Rab11b is expressed in the heart, brain and testes and functions in recycling of molecules in polarized cells (Silvis et al., 2009). EBV transmission was partially inhibited by a single knockdown of Rab11 isoforms, and synergistically inhibited by knockdown of both isoforms (Fig. 6e, f). These results taken together indicate that cell-to-cell EBV transmission occurred in a Rab11-dependent manner, likely mediated by translocation of ICAM-1 to the cell surface.

**Cell-to-cell contact facilitates clathrin-dependent endocytosis in the recipient cells, which contributes to infection of EBV**

Previously we demonstrated that cell contact induces the viral lytic cycle in the donor cells (Nanbo et al., 2012); however, little is known about the mechanism by which newly generated EBV virions enter Vero-E6 cells. To illuminate the mechanism that is involved in this process, we assessed the role of endocytosis in EBV transmission. We performed EBV transmission assays in the presence of dynasore (Newton et al., 2006), which is a specific inhibitor for dynamin, a large GTPase that plays an essential role in vesicle scission during clathrin- and caveolea-dependent endocytosis (Orth et al., 2002). Treatment with dynasore suppressed EBV transmission in a dose-dependent manner (Fig. 7a). We also transiently expressed a wt dynamin 2 (wtDyn2) and a dominant-negative form of dynamin 2 that has an amino acid substitution (Dyn2 K44A) (Orth et al., 2002) in Vero-E6 cells (Fig. 7b), and assessed their effect on EBV transmission. The expression of wtDyn2 upregulated viral transmission (Fig. 7c). In contrast, the expression of Dyn2 K44A suppressed viral transmission (Fig. 7c). These results indicate that EBV was transmitted to Vero-E6 cells through dynamin-dependent endocytosis. Treatment with NH4Cl, which blocks acidification of endosomes, also interfered with viral transmission (Fig. 7d), indicating that EBV virions subsequently fuse in endosomal compartments in a low-pH-dependent manner. Previously we demonstrated that the lytic cycle is induced in Akata+ cells by co-culture with Vero-E6 cells. To exclude the possibility that the inhibitors inhibited EBV replication in co-cultured B cells, we analysed the effect of these inhibitors on the expression of the viral lytic gene, gp350 (Maruo et al., 2001b; Nemerow et al., 1987) by flow cytometric analysis. The expression of gp350 was enhanced under the treatment of αhlgG (Fig. 7e). Treatment with 1 µM dynasore or 20 mM NH4Cl did not impair αhlgG-induced gp350 expression (Fig. 7e), indicating that these inhibitors specifically block the endocytosis-mediated internalization process in recipient cells.

To identify the endocytic pathway for EBV transmission, we examined the effect of inhibiting the clathrin heavy chain (CHC), Cav1, or sorting nexin 1 (SNX1) in Vero-E6 cells with siRNA. CHC, Cav1 and SNX1 play roles in clathrin-, caveolea- and macropinocytosis-mediated internalization, respectively (Kerr et al., 2006; Manninen et al., 2005; Moskowitz et al., 2005). Downregulation of CHC expression significantly suppressed EBV transmission (Fig. 8a, b), indicating that clathrin-mediated endocytosis likely contributes to EBV transmission. However, viral transmission was not blocked by downregulation of Cav1 and SNX1 (Fig. 8b), further supporting the conclusion that caveolea-mediated endocytosis and macropinocytosis were not important for EBV transmission. Finally, we examined whether direct cell contact enhances clathrin-mediated endocytosis in Vero-E6 cells. Akata+ and Akata− cells were co-cultured with Vero-E6 cells for 6 h in the presence of fluorescently labelled transferrin, a specific ligand of the clathrin-mediated pathway. Uptake of transferrin was enhanced in Vero-E6 cells that were co-cultured with EBV-positive and -negative Akata cells (Fig. 8d). Furthermore its uptake was suppressed by downregulation of CHC in Vero-E6 cells (Fig. 8c, d). The data indicate that cell–cell contact upregulates clathrin-mediated endocytosis in recipient cells, leading to internalization of progeny EBV replicated in donor cells.

**DISCUSSION**

Here, we show that EBV exploits host endocytic machinery to establish successful cell-to-cell transmission into epithelial cells. By use of a co-cultivation system with BL cells and various epithelial cells, we demonstrated that (i) a variety of adhesion molecules are expressed in EBV-positive and -negative Akata cells, and multiple epithelial cells (Fig. 1); and the interaction between β1 and β2 integrins and their ligands contributes to viral transmission (Fig. 2); (ii) multiple adhesion molecules are upregulated in co-cultured BL cells (Fig. 3); (iii) ICAM-1 distributes to the plasma membrane in co-cultured BL cells (Fig. 4) in a recycling endosome-dependent manner (Figs 5 and 6); and transmission of EBV virions into epithelial cells is mediated by clathrin-dependent endocytosis, which is accelerated by co-cultivation (Figs 7 and 8).

In the VS-mediated retrovirus transmission, the interaction between LFA-1 (αLβ2) in the recipient CD4+ T cells and
Fig. 6. Role of recycling endosomes in the transmission of EBV mediated by cell-to-cell contact. (a) Expression of wtRab11 and a dominant-negative form of Rab11 (Rab11 N25S) in Akata EBV-eGFP cells. eGFP-fused wtRab11 or Rab11 N25S was expressed in Akata EBV-eGFP cells with a retrovirus vector. As a control, Akata EBV-eGFP cells were transduced with a retrovirus vector encoding the eGFP gene. The expression of eGFP-wtRab11 or -Rab11 N25S was analysed by Western blot at 48 h post-infection (top). As an internal control, the expression of β-actin is shown (bottom). (b) Effect of expression of eGFP-Rab11 N25S on the expression of ICAM-1 in donor cells. eGFP-fused wtRab11 or Rab11 N25S was expressed in Akata EBV-eGFP cells with a retrovirus vector. The expression of ICAM-1 in Akata eGFP cells expressing wtRab11 (boldface line) or Rab11 N25S (thin line) was analysed by flow cytometry. As a control, Akata EBV-eGFP cells were transduced with a control plasmid (dashed line). Representative peak are shown. (c) Effect of expression of eGFP-Rab11 N25S on the distribution of ICAM-1 in donor cells. eGFP-fused wtRab11 or Rab11 N25S was expressed in Akata EBV-eGFP cells with a retrovirus vector. The expression of ICAM-1 in Akata EBV-eGFP was analysed by flow cytometry. The data are normalized to the expression of ICAM-1 in the cells without cell contact. The experiment was performed three times independently and the mean values and their sd are shown for each condition. **P<0.01 versus respective control (Student’s t-test). (d) Effect of expression of eGFP-Rab11 N25S on the transmission of EBV mediated by cell-to-cell contact. eGFP-fused wtRab11 or Rab11 N25S was expressed in Akata EBV-eGFP by a retrovirus vector. At 48 h post-infection, Akata EBV-eGFP cells were co-cultured with Vero-E6 for 24 h. The percentages of eGFP-expressing Vero-E6 cells were analysed by flow cytometry. The data are
ICAM-1 in virally infected dendritic cells (Gilbert et al., 2007; Groot et al., 2006; Gummuluru et al., 2002; Wang et al., 2009) and T cells (Hioe et al., 2001; Jolly et al., 2004, 2007; Tardif & Tremblay, 2003, 2005) is important for viral transmission. In contrast, little is known about the role of β1 integrin in the VS. Our data indicate that both β1 and β2 integrins and their ligands are expressed in BL cells and epithelial cells (Fig. 1) and that their interaction contributes to EBV transmission (Fig. 2), suggesting that a bidirectional interaction of multiple adhesion molecules leads to efficient EBV transmission. We observed that individual blocking antibodies for various adhesion molecules suppressed viral transmission moderately (Fig. 2), suggesting that multiple adhesion molecules may contribute to it in a complementary fashion.

We observed that direct cell contact upregulated several adhesion molecules in BL cells (Fig. 3e, g). In particular, ICAM-1 rapidly distributed to the plasma membrane of Akata cells even within a 1 h long co-cultivation (Fig. 4a, c, e, f), which is unlikely to be mediated by de novo synthesis of ICAM-1. Moreover, a fraction of ICAM-1 partially co-localized with Rab11 in the cytoplasm (Fig. 5). We also demonstrated that blocking Rab11 function by either overexpression of a dominant-negative form of Rab11 or knockdown of Rab11 suppressed both the translocation of ICAM-1 to the cell surface of donor cells and EBV transmission (Fig. 6).

Earlier studies demonstrated that Rab11 regulates transport of a wide variety of adhesion molecules including LFA-1, α5β1 integrin, neural cell adhesion molecule (NCAM) and E-cadherin (Desclozeaux et al., 2008; Diestel et al., 2007; Kelly et al., 2012; Lock & Stow, 2005; Mamdouh et al., 2003; Welz et al., 2014). Consistent with our result (Fig. 5), some recycling neural cell adhesion molecule and E-cadherin have been found to co-localize with Rab11 in the cytoplasm (Desclozeaux et al., 2008; Kelly et al., 2012; Lock & Stow, 2005). ICAM-1 appears to be recycled to the cell surface, although the involvement of Rab11 in this trafficking has not been established (Muro et al., 2005). Our data indicate that EBV likely exploits the Rab11-mediated recycling of ICAM-1 for further stabilization of cell contact and subsequent viral transmission. In addition, the pattern of distribution of ICAM-1 in co-cultured BL cells (Figs 4 and 5) was distinct from the tight distribution formed in the

Previously, Shannon-Lowe and colleagues investigated the mechanism of transmission by cell-to-cell contact of EBV. In contrast to our co-culturing system, they adsorbed cell-free EBV virions to the surface of EBV-negative B cells, and co-cultured these cell-bound virions with epithelial cells. They demonstrated that the majority of virions were retained on cell surfaces and that co-cultivation with epithelial cells initiated formation of CD21-mediated VS-like conjugate at the interface with contacted cells, leading to efficient EBV infection (Shannon-Lowe & Rowe, 2011; Shannon-Lowe et al., 2006). The same group observed that the site of conjugation was composed of viral glycoproteins, viral receptors derived from B cells and a variety of adhesion molecules (Shannon-Lowe & Rowe, 2011).

Multiple observations indicate that epithelial cells are likely infected in vivo during their intimate contact with EBV-positive B cells, a situation we reproduce with our co-culture condition. For example, it has been demonstrated that the mucosa of oropharyngeal tissue is heavily infiltrated by lymphocytes (Nagura, 1992) a site where EBV transmission is thought to occur. In addition, the atrophic border of the gastric mucosa, where EBV-associated gastric carcinoma develops, frequently generates mild-to-moderate atrophy and attracts inflammatory cells including lymphocytes (Hirano et al., 2003). We have demonstrated that cell-to-cell contact induces the viral lytic cycle in EBV-infected BL cells (Nanbo et al., 2012), indicating that it is likely that cell contact between infiltrating B cells and epithelial cells initiates the lytic cycle in B cells, which would promote the establishment of viral transmission. In accord with this idea, the lytic cycle in EBV-infected cells has been observed in secondary lymphoid tissues (Hudnall et al., 2005).

These observations, along with pathological studies, suggest that transmission of EBV by cell-to-cell contact between latently infected B cells and epithelial cells is an appropriate model for the establishment of persistent EBV infection in oropharyngeal tissue and/or for development of EBV-associated gastric cancer.

Several studies indicate that the direct interaction between viral glycoproteins gH–gL complex and the αvβ5, αvβ6 and...
αvβ8 integrins initiates fusion of cell-free EBV and the plasma membrane of epithelial cells (Chesnokova & Hutt-Fletcher, 2011; Molesworth et al., 2000). In contrast, Tugizov et al. (2003) demonstrated that cell-free EBV enters polarized oral epithelial cells through bidirectional transcytosis, mediated either by macropinocytosis from apical to basolateral, or

Fig. 7. Role of dynamin-dependent endocytosis in cell-to-cell EBV transmission. (a) Effect of dynasore on cell-to-cell EBV transmission. Vero-E6 cells were pretreated with various concentrations of dynasore for 30 min at 37 °C. Akata-EBV-eGFP cells were co-cultured with Vero-E6 cells for 24 h in the presence of dynasore. (b) Expression of wtDyn2 and a dominant negative form of Dyn2 (Dyn2 K44A) in Vero-E6. wtDyn2-eGFP or Dyn2 K44A-eGFP were expressed in Vero-E6 cells. As a control, Vero-E6 cells were transfected with pEGFP-N1 plasmid. The expression of wtDyn2-eGFP or Dyn2 K44A-eGFP was analysed with a Western blot at 48 h post infection (top). As an internal control, the expression of β-actin is shown (bottom). (c) Effect of expression of wtDyn2-eGFP and Dyn2 K44A-eGFP on EBV transmission mediated by cell-to-cell contact. Vero-E6 cells were transfected with expression plasmids for wtDyn2-eGFP and Dyn2 K44A-eGFP. At 48 h post-transfection, Vero-E6 cells were co-cultured with Akata-EBV-eGFP cells for 24 h. (d) Effect of NH₄Cl on EBV transmission mediated by cell-to-cell contact. Vero-E6 cells were pretreated with various concentrations of NH₄Cl for 30 min at 37 °C. Akata-EBV-eGFP cells were treated with DMSO, dynasore or NH₄Cl for 24 h. The percentages of eGFP-expressing Vero-E6 cells were analysed by flow cytometry. The data are normalized to eGFP expression in co-cultured Vero-E6 cells that were treated with DMSO (a, d) and that were transfected with a control plasmid (c). The experiment was performed three times independently, and the mean values and their SD are shown for each condition. *P<0.05 versus respective control. **P<0.01 versus respective control (Student’s t-test). (e) Effect of the inhibitors on EBV replication. Akata-EBV-eGFP cells that were treated or not treated with αIgG were incubated with DMSO, dynasore or NH₄Cl for 24 h. The expression of gp350 was analysed by flow cytometry. The data are normalized to αIgG-untreated and DMSO-treated cells (bottom). The experiment was performed three times independently, and the mean values and their SD are shown for each condition. ** P<0.01 versus respective control. NS, not significant.
by caveolae-mediated endocytosis from basolateral to apical surfaces. These contrasting findings reveal that cell-free EBV enters epithelial cells via multiple pathways, which may reflect different receptors and cell types. Previously an EBV glycoprotein, BMRF2, was identified as a viral factor that contributes to cell-to-cell EBV transmission through interaction with

**Fig. 8.** Role of clathrin-dependent endocytosis in EBV transmission mediated by cell-to-cell contact. (a) Efficiency of knockdown of target genes in Vero-E6 cells. Vero-E6 cells were transfected with control, CHC, Cav1 or SNX1 siRNA. The downregulation of individual genes was analysed by flow cytometry at 48 h post-transfection. The data are normalized to the expression of individual genes in Vero-E6 cells that were transfected with control siRNAs. The experiment was performed three times independently, and the mean values and their SD are shown for each condition. **P<0.01 versus respective control (Student’s t-test). (b) Effect of knockdown of target genes in Vero-E6 cells on EBV transmission mediated by cell-to-cell contact. Vero-E6 cells were transfected with control, CHC, Cav1 or SNX1 siRNAs. At 48 h post-transfection, the Vero-E6 cells were co-cultured with Akata− EBV-eGFP cells for 24 h. The percentages of eGFP-expressing Vero-E6 cells were analysed by flow cytometry. The data are normalized to the expression of eGFP Vero-E6 cells that were transfected with a control siRNA. The experiment was performed three times independently, and the mean values and their SD are shown for each condition. **P<0.01 versus respective control (Student’s t-test). (c) Knockdown efficiency of CHC in Vero-E6 cells. Vero-E6 cells were transfected with a control or CHC siRNA. The knockdown of the target gene was analysed by flow cytometry at 48 h post-transfection. The data are normalized to expression of CHC in Vero-E6 cells that were transfected with a control siRNA. The experiment was performed three times independently, and the mean values and their SD are shown for each condition. **P<0.01 versus respective control (Student’s t-test). (d) Effect of cell contact on clathrin-mediated endocytosis in Vero-E6 cells. Vero-E6 cells were transfected with control or CHC siRNAs. At 48 h post-transfection, the Vero-E6 cells were co-cultured with Akata+ or Akata− cells for 6 h at 37 °C. The co-cultured cells were further incubated in the presence of 2 μg ml⁻¹ Alexa Fluor 594-transferrin for 10 min at 37 °C. The Vero-E6 cells were harvested by trypsin and fixed with 4 % paraformaldehyde. The uptake of Alexa Fluor 594-transferrin was analysed by flow cytometry. The data are normalized to the uptake of 594-transferrin in Vero-E6 cells that were transfected with a control siRNA without co-cultivation. The experiment was performed three times independently, and the mean values and their SD are shown for each condition. **P<0.01 versus respective control (Student’s t-test).
α5β1, α3β1 and αvβ1 integrins (Xiao et al., 2008, 2009). However, other studies indicate that BMRF2 is not required for cell-to-cell fusion (Haan et al., 2001; McShane & Longnecker, 2004) and apparently very few BMRF2 molecules exist on the virion (Johannsen et al., 2004), suggesting that undefined additional receptors in epithelial cells may function in the transmission of EBV mediated by cell contact.

We demonstrated that EBV is likely transmitted to Vero-E6 cells via clathrin-dependent endocytosis, which is facilitated by cell-to-cell contact (Figs 7 and 8). We have also demonstrated that the neutralization antibodies for αvβ3 and αvβ6 did not block the transmission of EBV mediated by cell-to-cell contact (Fig. 2). Thus, it is unlikely that EBV transmission is established by fusion between the virus envelope and the cell membrane.

We favour a model for the transmission of EBV by cell-to-cell contact, in which the initial cell contact is established by the interaction of pre-existing adhesion molecules in BL cells and epithelial cells, resulting in the recruitment of additional adhesion molecules to the cell surface of BL cells followed by stabilization of the cell contact. The initial cell contact between BL cells and epithelial cells also upregulates clathrin-dependent endocytosis in recipient cells, which in turn facilitates the delivery of virions to the recipient cells. Taken together, our study demonstrates that EBV exploits the host endocytic pathway for efficient viral transmission, providing new insights into the mechanism of cell-to-cell viral transmission.

METHODS

Plasmids. The pEGFP-C3 plasmids encoding eGFP-fused wtRab11 (eGFP-wtRab11) and dominant-negative form Rab11 (eGFP-Rab11 S25N) (Chen et al., 1998) were kind gifts from Dr Angela Wandinger-Ness (University of New Mexico). The eGFP-wtRab11 and eGFP-Rab11 S25N cDNAs were cloned into a Moloney murine leukemia virus-based retrovirus plasmid (Kenney, 2007), a kind gift from Dr Bill Sugden (University of Wisconsin-Madison). The pEGFP-N1 plasmids encoding eGFP-fused wt dynamin 2 (wtDyn2-eGFP) and dominant-negative form dynamin (Dyn2 K44A-eGFP) (Orth et al., 2002) were kind gifts from Dr Mark A. McNiven (Mayo Clinic, Rochester, Minnesota, USA).

Cell culture. EBV-positive and -negative African BL-derived Akata cells (Akata and Akata, respectively) (Sanbo et al., 2002; Shimizu et al., 1994; Takada et al., 1991) were maintained in RPMI-1640 medium containing 10% FBS (Sigma-Aldrich) and antibiotics. Akata EBV-eGFP cells, which are latently infected with a recombinant Akata strain EBV encoding eGFP gene inserted into the viral BXLFI ORF (Maruo et al., 2001a), were maintained in RPMI-1640 medium containing 10% FBS, antibiotics and 800 μg/ml G418. EBV-negative African green monkey kidney epithelial Vero-E6 cells (Desmyter et al., 1968; Nanbo et al., 2010, 2012, 2013b), which were provided by Dr Ayato Takada (Hokkaido University), human gastric carcinoma epithelial AGS cells (Barranco et al., 1983; Yoshitama et al., 1997) and human nasopharyngeal carcinoma CNE1 cells (Suzhong et al., 1983) were grown in high-glucose Dulbecco’s modified Eagle’s medium containing 10% FBS and antibiotics. Cells were maintained at 37°C in 5% CO2.

Analysis of expression of adhesion molecules. For analysis of expression of adhesion molecules, Akata, Akata, Vero-E6, AGS or CNE1 cells (5 x 10^5 each) were incubated with mouse mAbs for CD29 or integrin β1 subunit (clone 4B4; Beckman Coulter), CD49d or integrin α4 subunit (clone HP12; Beckman Coulter), CD49e or integrin α5 subunit (clone SAM1; Beckman Coulter), CD11a or integrin αL subunit (clone EP1285Y; Abcam), CD18 or integrin β2 subunit (clone 10E12; Abcam), VCAM-1 (clone STA; Abcam), fibronectin (clone IST-3; Sigma-Aldrich), ICAM-1 (clone Ab-2; Sigma-Aldrich), αvβ5 (clone P5H9; Beckman Coulter) or αvβ6 (clone 10D5; Abcam) for 1 h on ice.

As a control, expression of ER was analysed with mouse anti-ER mAb (clone TE111.5D11; Thermo Scientific). The cells were washed twice in PBS and incubated with Alexa Fluor 488-labelled goat anti-mouse IgG (Life Technologies) for 30 min on ice. After washing twice in PBS, the expression of individual molecules was analysed by flow cytometry. For the analysis of expression of adhesion molecules in co-cultured cells, Akata, Akata or Akata EBV-eGFP cells (2 x 10^5) were co-cultured with Vero-E6 cells (2 x 10^5) in six-well plates for 6 h. After removal of the supernatant containing unbound cells, Akata cells that were still attached to Vero-E6 cells were removed by gentle pipetting. Vero-E6 cells were harvested by trypsinization. The expression of adhesion molecules in Akata cells and Vero-E6 cells was analysed by flow cytometry as described above. In parallel, the harvested cells were analysed by flow cytometry with PE-labelled mAb for HLA-DR (Sigma-Aldrich) and rabbit polyclonal antibody for Cav1 (Abcam) to confirm that harvested cells did not contain co-cultured cells.

EBV-transmission assay. Vero-E6, AGS or CNE1 cells (5 x 10^5) were co-cultured with Akata EBV-eGFP cells (5 x 10^5) for various times in 24-well plates in the presence or absence of 1% goat anti-human IgG (Dako). To remove the co-cultured Akata EBV-eGFP cells, the epithelial cells were washed with the medium three times, trypsinized and cultured in six-well plates for 6 h. Vero-E6 cells were harvested and fixed in 4% paraformaldehyde (PFA) in PBS for 10 min at room temperature. The percentages of eGFP-positive epithelial cells were analysed by flow cytometry (FACS Calibur; Becton, Dickinson and Company). In parallel with flow cytometric analysis, the same sample was analysed by confocal laser scanning microscopy to confirm that the sample did not contain Akata EBV-eGFP cells. To examine the effect of blocking antibodies to adhesion molecules on EBV transmission, Akata EBV-eGFP cells and individual epithelial cells were separately pre-incubated with mouse mAbs for CD29 (clone 4B4) (Martinez-Vitambres et al., 2012), CD49d (clone HP12) (Porter & Hogg, 1997), CD49e (clone SAM1) (Porter & Hogg, 1997), VCAM-1 (clone B-K9; Abcam) (Lefevre et al., 2009), ICAM-1 (clone BBIG-11; Beckman Coulter) (Peninno et al., 2010), αvβ5 (clone P5H9) (Echevarria et al., 2011), αvβ6 (clone 10D5) (Huang et al., 1998) or ER (clone TE111.5D11) (Biswas et al., 1998) for 30 min at 37°C. Pretreated cells were co-cultured in the presence of individual antibodies for 24 h and EBV transmission into epithelial cells was analysed by flow cytometry. To determine the role of dynamin in EBV transmission, Vero-E6 cells were transfected with the expression plasmids for wtDyn2-eGFP or Dyn2 K44A-eGFP with TransIT-LT1 (Mirus). At 48 h post-transfection, the cells were co-cultured with Akata EBV-eGFP cells for 24 h and EBV transmission was described above. The expression of Dyn2 derivatives was analysed by Western blot with rabbit anti-dynamin polyclonal antibody (clone C2C3; GeneTex).

Immunofluorescent staining. Akata or Akata cells (2 x 10^5) were co-cultured with Vero-E6 cells (2 x 10^5) in 35 mm glass-bottomed culture dishes (MatTek Corporation) for various times. The cells were fixed with 4% PFA in PBS for 10 min at room temperature, permeabilized with PBS containing 0.05% Triton X-100 for 10 min at room temperature and blocked in PBS containing 1% BSA and 0.05% Triton X-100 for 20 min at room temperature. The cells were incubated with mouse anti-ICAM-1 mAb (clone Ab-2) and/or rabbit anti-Rab1 polyclonal antibody (Abcam) for 1 h at room temperature. After washing twice in PBS, the cells were incubated with Alexa Fluor 488-labelled anti-mouse IgG and/or Alexa Fluor 594-labelled anti-rabbit IgG (Life Technologies).
for 1 h at room temperature. After washing twice in PBS, the nuclei were counterstained with DAPI. Images were collected with a ×60 water-immersion objective (numerical aperture=1.3) of a confocal laser scanning microscope (FluoView FV10i; Olympus) and acquired by using FV10-ASW software (Olympus).

**Retroviral infection.** Recombinant retroviruses for the expression of eGFP-wtRab11 and eGFP–Rab11 S25N were produced and purified as previously described (Nanbo et al., 2002, 2010, 2012). For retroviral infections, Akata+ EBV-eGFP cells (1×10⁶) were grown in 24-well plates, at which point the culture medium was replaced with ice-cold MEM supplemented with 10% FBS and 20 mM HEPES (pH 7.4), and the cells were incubated with viral stocks (10⁻⁷–10⁻⁶ infectious units ml⁻¹) for 1 h at 4°C at an m.o.i. of 5. After being washed twice with complete medium, the cells were cultured in complete medium for 48 h. The expression of eGFP-wtRab11 and eGFP-Rab11 S25N was analysed by Western blotting with mouse monoclonal anti-Rab11 antibody (clone C14, Abcam). Downregulation of the Rab11 gene expression (0.75 kV, 3 ms; Nepa Gene). Downregulation of the Rab11 gene expression was analysed by Western blotting with mouse monoclonal anti-Rab11 antibody (clone 47/Rab11; Beckman Coulter) at 48 h post-infection. The effect of expression of eGFP-wtRab11 and eGFP-Rab11 S25N on EBV transmission into Vero-E6 cells was analysed as described for the EBV transmission assay.

**siRNA treatment.** Target sequences corresponding to the human Rab11a (Takahashi et al., 2012), Rab11b (Moskowitz et al., 2005), CHC, Cav1 (Manninen et al., 2005) and SNX1 (Kerr et al., 2006; Mamdouh et al., 2003) coding sequences were selected and synthesized (Life Technologies). As control, siRNAs with the target sequence against human glyceraldehyde-3-phosphate dehydrogenase (Shanghai GenePharma) were used (Nanbo et al., 2013a). siRNAs against Rab11a and/or Rab11b were transfected into Akata+ EBV-eGFP cells (1×10⁶) by electroporation (0.75 kV, 3 ms; Napa Gene). Downregulation of the Rab11 gene was analysed by Western blotting with mouse monoclonal anti-Rab11 antibody (clone 47/Rab11) at 48 h post-transfection. CHC, Cav1 or SNX1 siRNA was transfected into Vero-E6 cells (1×10⁶) by using TransIT-TKO (Takara Bio). The downregulation of individual target genes was analysed by flow cytometric analysis with rabbit anti-CHC, anti-Cav1 or anti-SNX1 polyclonal antibody (all from Abcam). The efficiency of knockdown of Rab11 isoforms in Akata+ EBV-eGFP cells, and that of CHC, Cav1 or SNX1 in Vero-E6 cells on EBV transmission was analysed as described for the EBV transmission assay.

**Inhibitor treatment.** To examine the effect of inhibitors on EBV transmission, Akata+ EBV-eGFP cells and Vero-E6 cells were separately pretreated with various concentrations of dynasore (Sigma-Aldrich) or NH₄Cl (Sigma-Aldrich) for 30 min. Pretreated cells were co-cultured in the presence of inhibitors for 24 h and EBV transmission was analysed as described above. For analysis of the effect of the inhibitors on induction of lytic cycle, Vero-E6 cells were co-cultured with Akata+ cells treated with 1% α-hIgG in the presence of 1 μM dynasore or 20 mM NH₄Cl for 24 h. Akata+ cells were harvested, fixed, permeabilized and blocked as described above. The cells were incubated with anti-gp350 mAb (C-1) (Thorley-Lawson & Geilinger, 1980) for 1 h at room temperature, washed twice in PBS and incubated with Alexa Fluor 488-labelled secondary antibody. After washing twice in PBS, the expression of gp350 was analysed by a flow cytometry.

**Transferrin uptake assays.** Akata+ or Akata– cells were co-cultured with control or CHC siRNA transfected Vero-E6 cells for 6 h at 37°C. Co-cultured cells were further incubated in the presence of 2 μg ml⁻¹ Alexa Fluor 488-labelled transferrin (Life Technologies) for 10 min at 37°C. Vero-E6 cells were harvested by trypsin and fixed with 4% PFA. The efficiency of uptake of transferrin was analysed by use of flow cytometry.

**ACKNOWLEDGEMENTS**

We acknowledge Drs Ayato Takada (Hokkaido University), Angela Wandinger-Ness (University of New Mexico), Mark A. McNiven (Mayo Clinic, Rochester, Minnesota, USA) and Bill Sugden (University of Wisconsin, Madison) with Vero-E6 cells, expression plasmids for eGFP-wtRab11 and eGFP-Rab11 S25N, expression plasmids for wtDyn2-eGFP and Dyn2 K44A-eGFP and a retrovirus plasmid, respectively. We also thank Dr Bill Sugden for critically reviewing the manuscript. This work was supported by grants-in-aid from the Japanese Ministry of Education, Culture, Sports, Science and Technology, Japan and Takeda Science Foundation.

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