Epstein–Barr virus-infected cells release Fas ligand in exosomal fractions and induce apoptosis in recipient cells via the extrinsic pathway

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Epstein–Barr virus (EBV; human herpesvirus 4) is an oncogenic herpesvirus implicated in the pathogenesis of several human malignancies. A number of recent studies indicate that EBV can manipulate the local microenvironment by excreting viral and cellular components in nanovesicles called exosomes. In this study, we investigated the impact of EBV-derived exosomes on apoptosis of recipient cells and the molecular pathway involved in this process. Exosomes from EBV-infected but not from non-infected cells induced apoptosis in a number of different cell types, including B-cells, T-cells and epithelial cells. However, this phenomenon was not universal and the Burkitt’s lymphoma-derived B-cell line BJAB was found to be resistant to apoptosis. Exosomes from both type I and type III EBV latently infected cells induced apoptosis in a dose- and time-dependent manner. Moreover, cells exposed to EBV exosomes did not form colonies in soft agar assays. We further show that fluorescently labelled exosomes derived from EBV-infected cells are taken up by non-infected cells and induce apoptosis via the extrinsic pathway. Inhibition of caspase-3/7/8 blocks EBV exosome-mediated apoptosis. Furthermore, our data indicate that EBV exosomes trigger apoptosis through the Fas ligand (FasL)-mediated extrinsic pathway, as FasL was present in EBV exosomal fractions and anti-FasL antibodies could block EBV exosome-mediated apoptosis. Together, these data support the notion that EBV hijacks the exosome pathway to excrete viral and cellular components that can modulate its microenvironment.

INTRODUCTION

Cells communicate with their surroundings by releasing a number of cellular factors. This cell-to-cell communication can result in a multitude of cellular processes, including cell growth, differentiation, migration and even immune modulation. In recent years, it has emerged that nanovesicles are key modulators of intercellular communication and transporters of biologically active compounds (Villanueva, 2014). These membrane-bound nanovesicles (known as exosomes) are ~30–150 nm in size and originate from the cell’s endosomal membrane system (Raposo & Stoorvogel, 2013). Exosomes have a typical cup- or dish-like morphology and are found to be secreted in body fluids, including blood, urine, saliva and breast milk (Inal et al., 2013). These nanovesicles are generated through inward budding of cytoplasmic endosomal-derived membranes of multivesicular bodies which are transported to the plasma membrane and subsequently released as exosomes (Colombo et al., 2014). Exosomes have been shown to carry a wide range of molecules, including mRNA, microRNA (miRNA), proteins and soluble factors, which can induce various cellular changes in target cells, including tumour development, invasion, metastasis and even apoptosis (Hood et al., 2011; Kim et al., 2005; Wieckowski et al., 2009; Yang et al., 2013). Moreover, the mechanism of exosome biogenesis has considerable overlap with the assembly and release of numerous enveloped viruses (Meckes, 2015), and a number of studies now indicate that some viruses can also hijack the exosome pathway to evade the immune system and to contribute to viral-associated pathogenesis (Izquierdo-Useros et al., 2010).

Epstein–Barr virus (EBV; human herpesvirus 4) is a large enveloped herpesvirus implicated in the pathogenesis of several human malignancies, including Burkitt’s lymphoma, Hodgkin’s lymphoma, nasopharyngeal carcinoma and gastric carcinoma (Longnecker et al., 2013). The virus infects and establishes long-term latency in B-lymphocytes (Khan et al., 1996; Sinclair & Farrell, 1995). In latently infected cells, the virus expresses one of four different patterns of gene expression, referred to as type 0, I, II or III latency (Young & Rickinson, 2004). Type 0 is characterized by the expression...
of EBV-encoded small RNAs (EBERs) only. This type of latency is believed to occur in healthy carriers. In type I latency, in addition to EBERs, Epstein–Barr nuclear antigen 1 (EBNA1) and BamHI A rightward transcripts (BARTs) are expressed. This form of latency is typically observed in Burkitt’s lymphoma and gastric carcinoma patients. In type II latency, EBNA1, EBERs, BARTs and latent membrane proteins (LMP1, LMP2A and LMP2B) are expressed, and it is seen in nasopharyngeal carcinoma and Hodgkin’s lymphoma. In latency type III, all of the known latent genes are expressed, including six EBNAs, two EBERs and three LMPs. This pattern of latency is typically seen in in vitro EBV-immortalized lymphoblastoid cell lines (LCLs) and post-transplant lymphoproliferative disorders (Kelly et al., 2006). A number of viral miRNAs have also been shown to be expressed in different latencies (Amoroso et al., 2011; Pratt et al., 2009; Marquitz et al., 2014). It is now generally accepted that some of these viral latent products play a key role in cellular processes such as cell proliferation, inhibition of apoptosis and immortalization. Moreover, a number of recent studies indicate that EBV can also manipulate the local microenvironment by excreting specific viral and cellular components in exosomes. For example, exosomes released from nasopharyngeal carcinoma cells have been shown to contain LMP1 and galectin 9, and to inhibit T-cell activation and proliferation (Keryer-Bibens et al., 2006). Similarly, LMP1-positive exosomes derived from LCLs inhibit the proliferation of PBMCs (Flanagan et al., 2003). A number of EBV-specific miRNAs have also been found to be present in LCL exosomes that suppress the expression of target genes in recipient dendritic cells (Pegtel et al., 2010; Verweij et al., 2013). In addition to miRNAs, our previous study showed that EBER1 and EBER2 are also part of the cargo released in exosomes of EBV-infected cells (Ahmed et al., 2014). A more recent study showed that exosomes isolated from EBV-transformed B-cells contain Fas ligand (FasL) and MHC class II molecules, and can induce cell death of antigen-specific T-helper cells (Klinker et al., 2014). However, the molecular mechanisms by which exosomes derived from EBV-infected cells are internalized and the possible role of exosomes in the phenotypic modulation of recipient cells are not fully understood.

In this study, we report that exosomes derived from both type I and type III EBV latently infected cells induce apoptosis in a dose- and time-dependent manner. We further show that fluorescently labelled exosomes derived from EBV-infected cells are taken up by non-infected cells and induce apoptosis via the extrinsic pathway involving FasL.

**RESULTS**

**Exosomes from EBV-infected cells induce cell death in a dose-dependent manner**

Exosomes released from EBV-infected cell lines were isolated using differential ultracentrifugation as described previously (Ahmed et al., 2014). Transmission electron microscopy (TEM) was used to analyse the purified exosomes. Nanovesicles ranging in size from 50 to 120 nm with the typical morphology of exosomes (cup or disk shape) were clearly visible (Fig. 1a). To further confirm the identity of isolated exosomes, we performed Western blotting for CD63 (Verweij et al., 2011) and flotillin (Okabayashi & Kimura, 2010), both of which are established markers of exosomes. Exosomes from EBV-infected and non-infected cells were clearly positive for both markers (Fig. 1b).

To determine the effect of exosomes released by EBV-infected cells on non-infected cells, we initially exposed 293T cells to serially diluted B95.8 exosomes for 24 h and subsequently measured cell viability using the Cell Titre-Glo viability assay. All assays were carried out in duplicates and experiments were repeated three times. The results consistently showed that exosomes released from EBV-infected B95.8 cells induced cell death of 293T cells in a dose-dependent manner (Fig. 1c1). Moreover, the effect was very potent; even at dilutions as low as 1 : 64, EBV exosomes decreased cell viability by ~20%. We next investigated if exosomes from EBV-infected cells had a similar impact on other cell types, in particular B-cells (BL30) and T-cells (Jurkat). The results clearly indicated that both cell types were indeed susceptible to EBV exosomes and underwent cell death in a dose-dependent manner (Fig. 1c2, c3). Furthermore, the effect of EBV exosomes on cell viability was not unique to B95.8 cells and exosomes released from an EBV-immortalized LCL (GK-LCL) could also induce a similar effect (Fig. 1c4). Although B95.8 and GK-LCL are very different cell lines, the former being of marmoset origin and the latter of human origin, they both have a similar viral gene expression profile (type III latency). Thus, the finding that the pattern of apoptosis induced by exosomes from these cell lines was similar suggested that the virus and not the cell of origin was central to the excretion of death-inducing exosomes. Interestingly, BJAB cells (an EBV-negative Burkitt’s lymphoma cell line) were totally resistance to apoptosis with either B95.8 or GK-LCL exosomes (data not shown). We are currently working on determining the mechanism of resistance of these cells.

**Exosomes from EBV-infected cells induce cell death in a time-dependent manner**

To determine if the effect of EBV exosomes was time-dependent, we performed a time-course experiment using the highest concentration of the exosomes (1 : 4 dilution). The data indicated that the induction of cell death by EBV exosomes was not immediate or rapid. In fact, cells appeared to proliferate in the first few hours after exposure to exosomes (Fig. 2a). Significant cell death was only apparent at ~24 h after exposure, reaching a maximum of ~50% after 48 h (Fig. 2a). Moreover, these results correlated with caspase-3/7 activity, which was more than threefold higher in exosome-treated cells as compared with untreated cells at
Fig. 1. Isolation of exosomes from EBV-infected cells and their impact on recipient cells. Exosomes were isolated using differential ultracentrifugation and examined using TEM. (a) Nanovesicles with typical cup-shaped morphology and size resembling exosomes were isolated from culture supernatants of EBV-infected cells of different origin (see Methods). Bar, 200 nm. (b) Western blot analysis for the exosomal markers flotillin and CD63 further confirmed the identity of these nanovesicles to be exosomes. (c) The impact of EBV exosomes from B95.8 cells was tested on (c1) epithelial cells (293T), (c2) B-cells (BL30) and (c3) T-cells (Jurkat) at doubling dilution. Dose-dependent cell death was observed in all three cell lines relative to untreated (PBS) control group (normalized to 100%). Exosomes from an EBV-immortalized LCL (GK-LCL) also induced similar dose-dependent cell death in 293T cells (c4). The data are expressed as mean ± SD of three independent experiments.
48 h (Fig. 2b). These findings indicated that EBV exosomes induce cell death by apoptosis and this is time-dependent.

Although we routinely isolated exosomes from 80 ml culture supernatants (0.5 × 10⁶ cells ml⁻¹) and resuspended the final pellet in 100 µl PBS, it is nevertheless inevitable that the concentration of exosomes will vary significantly from batch to batch. Thus, 1 : 4 dilution of exosomes from one batch of cells may not be the same as 1 : 4 dilution from another batch. This could have a profound effect on downstream experiments. To limit this variability as much as possible, we decided to standardize our methodology by pooling several exosome isolates and performed the Bradford assay on all extracts. The protein concentration of the 1 : 4 diluted exosomes used in the above experiments was found to be equivalent to ~10–15 µg. Based on this, all subsequent apoptosis experiments were performed by exposing cells to 12.5 µg exosomes for 48 h in 100 µl reactions.

**EBV exosomes from both type I and type III latently infected cells induce apoptosis in 293T cells**

As the pattern of EBV gene expression is very different in type I and type III latently infected cells, it is possible that the exosomes released from these cells could also have a different impact on recipient cells. To address this, we isolated exosomes from Namalwa cells (type I latency) and GK-LCL (type III latency), and examined the effect of these exosomes on apoptosis of 293T cells. The results showed that exosomes from both cell types induced apoptosis (Fig. 3a) and this correlated with the caspase-3/7 activity (Fig. 3b). The level of apoptosis and the corresponding caspase-3/7 activity was greater in cells exposed to exosomes from type III cells (GK-LCL) compared with type I cells (Namalwa). Western blot analysis for LMP1 clearly indicated that this EBV protein was expressed in type III latently infected cells (LCL and B95.8) and their corresponding exosomes, but not in type I latently infected cells (Namalwa) and EBV-negative B-cells (BL30) and their exosomes (Fig. 3c). As exosomes from both type I and type III cells induced apoptosis of 293T cells, LMP1 does not appear to be the main trigger of this effect.

**EBV exosomes inhibit 293T cell colony formation in soft agar assays**

To check if the exosomes released by EBV-infected cells could inhibit the formation of colonies in soft agar, we exposed 293T cells to exosomes from EBV-infected and non-infected cells in soft agar assays. For the negative control, PBS was added to the wells instead of exosomes. After 7 days incubation, colonies were clearly visible in the negative control, but no such colonies could be seen from cells exposed to EBV exosomes (Fig. 4a). After 14 days incubation, these differences were even more apparent. Untreated cells formed colonies that were visible with the naked eye, whilst most of the cells exposed to EBV exosomes died and failed to form any colonies (Fig. 4b). We repeated the experiment and the same results were obtained. To test if EBV exosomes had any impact on established colonies, we repeated the experiment, but added EBV exosomes to 5-day-old established 293T cell colonies. Plates were incubated for a further 5–14 days and examined. We did not see
any clear difference in the number or size of the colonies in exosome-treated and non-treated colonies, suggesting that established cell colonies are not susceptible to EBV exosome-mediated apoptosis (data not shown).

**EBV exosomes are taken up by non-infected cells**

To understand the mechanism by which EBV exosomes were inducing cell death, we exposed 293T cells to 12.5 μg PKH67-labelled exosomes from GK-LCL and B95.8 cells. For negative controls, PKH67/PBS and PBS alone were used. DAPI was used as nuclear counterstain. The cells were incubated for 24 h with either labelled exosomes or controls and examined using fluorescent microscopy. It was observed that the exosomes from both cell types (B95.8 and GK-LCL) were taken up by 293T cells and were localized in the cytoplasm of the recipient cells (Fig. 5). No such staining was seen in the PBS negative control groups.

**Exosomes from EBV-infected cells, but not from non-infected cells, induce apoptosis via the induction of the caspase-3/7/8 pathway**

To further understand the mechanism of cell death induced by EBV exosomes, we purified exosomes from an EBV-negative and its subtype EBV-positive Burkitt’s lymphoma cell line, BL30 and BL30-B95.8, respectively. When 293T cells were exposed to 12.5 μg purified exosomes, apoptosis was observed only in cells that were exposed to exosomes from EBV-infected cells, but not from the non-infected counterpart (Fig. 6a). We have previously shown that EBERs are excreted from EBV-infected and EBER-transfected cells via exosomes (Ahmed et al., 2014). To determine if EBERs could be one of the viral components responsible for apoptosis, we isolated exosomes from EBER1-transfected 293T cells (ER1) and their counterpart vector-only-transfected cells (pHEBo) and subsequently examined their effect on 293T cells. We did not see any significant difference in apoptosis compared with the control (Fig. 6a). In fact, EBER1-containing exosomes appeared to promote cell proliferation rather than apoptosis. To determine the molecular pathway involved in EBV exosome-mediated apoptosis, we examined the activation of caspase-3/7, 8 and 9 (Fig. 6b–d). The cell viability data correlated with a significant increase in caspase-3/7 and 8 levels, often two- to threefold in EBV exosome-treated cells compared with controls (cells exposed to PBS instead of EBV exosome). Caspase-9 activation, however, was only moderately raised, indicating that EBV exosomes induce apoptosis predominantly via the activation of the extrinsic pathway.

**Fig. 3.** EBV exosomes from both type I and type III latently infected cells induce apoptosis. 293T cells were exposed for 48 h to 12.5 μg exosomes isolated from EBV type I latency (Namalwa) and type III latency (GK-LCL). (a) Exosomes from both type I and type III latently infected cells induced apoptosis of 293T cells. (b) The level of cell death correlated with that of caspase-3/7 activity. (c) Western blot analysis indicated the presence of LMP1 in type III latently infected cells (LCL and B95.8) and their exosomes, but not in type I cells (Namalwa) or non-infected cells (BL30 and 293T) and their exosomes. LMP1 was detected using a pool of four mAbs (CS1–4) directed to the C terminus of LMP1. The predicted molecular mass using these antibodies was 42 kDa, most probably representing a degraded form of LMP1. Exo, Exosome.
Inhibition of the caspase-3/7/8 pathway blocks apoptosis induced by EBV exosomes

To verify that exosomes from EBV-infected cells induced apoptosis via the extrinsic pathway, we blocked caspase-3/7/8 activity in 293T cells by incubating them with caspase inhibitor (CAS 210344-95-9) for 30 min before exposing them to 12.5 mg exosomes from EBV-positive (BL30-B95.8) and EBV-negative (BL30) cells. The results revealed a significant reduction in apoptosis of cells treated with caspase inhibitor (Fig. 7a). As exosomes from EBV-negative cells (BL30) did not induce apoptosis, the presence or absence of the caspase inhibitor had no impact on cell viability (Fig. 7a). This observation is also reflected in the activity of caspase-3/7 and 8 (Fig. 7b, c).

FasL is present in EBV exosomal fractions and blocking it inhibits apoptosis induced by EBV exosomes

Several studies have reported the presence of FasL in exosomes excreted by tumour cells (Andreola et al., 2002; Kim et al., 2005; Stenqvist et al., 2013). To determine if EBV immortalized cells also excreted FasL-carrying exosomes, we isolated exosomes from EBV-infected (latency type I and III) and non-infected cells and performed Western blotting for FasL. FasL was present in exosomal fractions isolated from all EBV-infected cell lines, irrespective of latency pattern, but absent from EBV-negative cell lines (Fig. 8a). It is noteworthy that a trimeric form of FasL with a molecular mass of ~80 kDa appeared to be present in EBV exosomes. An additional band at ~26 kDa was also occasionally seen in exosomal fractions from EBV-infected cells (data not shown). These findings are consistent with previous reports demonstrating that the trimeric form is the active and excreted form of FasL (Holler et al., 2003; Tanaka et al., 1995; Stenqvist et al., 2013). To test if FasL was responsible for EBV exosome-mediated apoptosis, we incubated 12.5 μg exosomes from EBV-positive (BL30-B95.8) and EBV-negative (BL30) cells with increasing concentrations of neutralizing anti-FasL mAb for 1 h and then exposed 293T cells to anti-FasL-treated and non-treated exosomes for 48 h. The results indicated that anti-FasL antibody could indeed block EBV exosome-mediated apoptosis in a dose-dependent manner (Fig. 8b). As in previous experiments, exosomes from EBV-positive cells, but not from EBV-negative cells, induced apoptosis of 293T cells and this could be blocked by anti-FasL antibody (Fig. 8c).
DISCUSSION

It is estimated that the global burden of deaths from EBV-associated malignancies is ~1.8% of all cancer deaths and this burden is predicted to increase with increasing incidence of cancer (Khan & Hashim, 2014). Thus, understanding the biology of this common herpesvirus and its mechanism(s) of pathogenesis is essential for any future interventions aimed at reducing or preventing EBV-associated malignancies. Exosomes derived from EBV-infected cells are emerging as important cellular factors in protecting infected tumour cells from destruction (Mrizak et al., 2015). Exosomes released from virally infected cells contain a number of viral and cellular factors that can induce different molecular and physiological changes in recipient cells (Klibi et al., 2009; Meckes et al., 2013; Mrizak et al., 2015; Nanbo et al., 2013). It has been shown that exosomes isolated from nasopharyngeal carcinoma and from EBV-immortalized LCLs have anti-proliferative or apoptotic effects on recipient cells (Flanagan et al., 2003; Keryer-Bibens et al., 2006; Klinker et al., 2014). The molecular mechanisms involved in these physiological changes are virtually unknown, but are believed to involve viral and cellular components such as LMP1, viral mRNA, and others.

![Figure 5](image_url)

**Fig. 5.** Exosomes released from EBV-infected cells are taken up by non-infected cells. 293T cells were exposed to PKH67-labelled exosomes from (a–c) GK-LCL and (d–f) B95.8. PBS (with and without PKH67 dye) was included as negative controls (g–i and j–l, respectively). DAPI was used as nuclear counterstain and slides were examined by fluorescence microscopy. Strong punctate cytoplasmic staining was seen in 293T cells exposed to GK-LCL and B95.8 exosomes (c, f). No such signals could be seen in PBS controls (i, l). Main bar, 50 μm.
viral miRNA or galectin excreted in exosomes from EBV-infected cells (Meckes, 2015). Which physiological effect dominates most probably depends on the cell type and relative amount of the exosomal cargo. Our previous study showed that EBV non-protein-coding small RNAs (EBER1 and EBER2) are also part of the exosomal cargo (Ahmed et al., 2014). What impact they exert on recipient cells is poorly understood.

The aim of this study was to determine the functional impact of exosomes released from EBV-infected cells and identify the potential molecular pathways involved. We hypothesized that EBV exosomes carrying viral components such as LMP1, LMP2 and EBERs would induce cell proliferation, probably involving the NFκB pathway (Meckes et al., 2013). To our surprise, when 293T cells were exposed to exosomes from EBV-infected cells, they underwent apoptosis. The impact of EBV exosomes on 293T cells was dose-dependent, with microgram levels of exosomes inducing as much as 50% cell death. Moreover, this potent apoptotic effect was also observed in B- and T-cells, and could be induced by exosomes derived from a variety of EBV-infected cells. As exosomes from non-infected cells did not induce apoptosis of recipient cells, this indicated that the exosomal cargo of EBV-infected cells was very different from non-infected cells, even in otherwise identical Burkitt’s lymphoma cell lines (BL30 and BL30-B95.8). Interestingly, the apoptotic effect of EBV exosomes was not immediate. In fact, in the first 6 h
after exposure of 293T cells to EBV exosomes, the cell viability gradually increased and significant apoptotic effect was only observed after 24 h exposure. These results corresponded with an increase in caspase-3/7 activity. Together, these observations implied that the apoptotic effect of EBV exosomes probably involved their uptake into recipient cells. This was further supported by our data showing that labelled exosomes are indeed taken up by recipient cells and induce apoptosis by triggering the extrinsic pathway. Furthermore, the observation that EBV exosomes prevented colony formation in soft agar assays also indicates that exosome uptake or at least close interaction with recipient cells is necessary.

What is/are the components within EBV exosomes triggering apoptosis? Our data show that exosomal fractions from both type I and type III EBV latently infected cells induce apoptosis in a dose-dependent manner involving the extrinsic pathway. In agreement with previous reports (Flanagan et al., 2003; Meckes et al., 2010), we also found LMP1 to be present in exosomes released from type III, but not type I, EBV latently infected cells. As exosomes from both types of latently infected cells induced apoptosis, it was clear that LMP1 was not the inducer of apoptosis observed in this study. Type I EBV latently infected cells typically express a very limited number of viral genes, i.e. EBNA1, EBERs and BARTs (Longnecker et al., 2013). The results of this study indicate that exosomes from EBER1-transfected cells do not induce apoptosis. On the contrary, EBER1-containing exosomes showed a moderate proliferative effect on recipient cells. This is consistent with a number of other studies reporting proliferative (Iwakiri et al., 2003; Yang et al., 2004) and anti-apoptotic roles for EBERs (Nanbo et al., 2002; Ruf et al., 2005). EBNA1 is a nuclear protein associated with EBV episome maintenance. There is no evidence that EBNA1 is excreted in exosomes from infected cells. Thus, EBNA1 is also unlikely to be the inducer of EBV exosome-mediated apoptosis. This leaves either BART miRNAs or some cellular component(s) excreted in exosomes. Over the past decade or so, a number of studies have shown that FasL is excreted in exosomes from a variety of different tumour cells and FasL-containing exosomes can induce apoptosis of recipient cells (Abrahams et al., 2003; Abusamra et al., 2005; Kim et al., 2005; Yang et al., 2013). Recently, Klinker et al. (2014) reported that EBV-immortalized LCLs also excrete FasL-containing exosomes which can induce apoptosis of CD4+ T-cells (Klinker et al., 2014). Our data confirms and extends the findings of Klinker et al. (2014). We show that FasL is present in exosomal fractions of both type I and type III EBV latently infected cells. Importantly, exosomal fractions from EBV-infected cells, but not non-infected cells, contained FasL and induced apoptosis via the activation of caspase-3/7/8. Furthermore, Fasl-mediated apoptosis could be inhibited by anti-human FasL antibodies in a dose-dependent manner. The expression of Fasl in EBV exosomal fractions was independent of LMP1 expression and FasL appeared to exist as a trimer of ~80 kDa in Western blots (Kayagaki et al., 1995; Tanaka et al., 1995; Stenqvist et al., 2013). Indeed, it has been reported that
most of the excreted form of FasL exists as a trimeric structure (Tanaka et al., 1995; Stenqvist et al., 2013) and two trimers held in close proximity are required for cytotoxic activity (Holler et al., 2003). Although we did not perform double immunoelectron microscopic studies to directly demonstrate the presence of FasL with exosomes in the current study, previous studies have shown that a biologically active form of FasL is indeed expressed in exosomes (Andreola et al., 2002; Klinker et al., 2014; Stenqvist et al., 2013). Furthermore, only the membrane-bound FasL and not the proteolytically processed soluble form of FasL appears to be capable of inducing apoptosis (Schneider et al., 1998; Suda et al., 1997; Tanaka et al., 1998).

The data presented here indicate that FasL is present in EBV exosomal fractions, most likely linked to exosomal membranes. The observation that anti-FasL antibodies can inhibit EBV exosome-mediated apoptosis suggests that FasL is the main apoptotic signal present in EBV exosomal fractions. Moreover, the detection of the 80 kDa membrane-bound, active form of FasL implies that the FasL released from EBV-infected cells is exosome-associated. However, the nature of the exosome isolation protocol is such that we cannot exclude the possibility of some contaminating factors such as TRAIL (TNF-related apoptosis-inducing ligand) that may be present in EBV exosomal fractions could also contribute to apoptosis (Stenqvist et al., 2013). Future studies aimed at directly demonstrating FasL with EBV exosomal membranes and the molecular interactions that occur between FasL-carrying exosomes and the recipient cells will help to further our understanding of how EBV modulates its microenvironment.

Which biological function of EBV exosomes, i.e. apoptosis (Klibi et al., 2009; Klinker et al., 2014), immune modulation (Flanagan et al., 2003; Meckes et al., 2010) or cell proliferation (Gutzeit et al., 2014; Nanbo et al., 2013), dominates is likely to depend on a number of factors, including type and state of the cells, EBV lytic or latent cycle, and the relative content of the exosomal cargo. For example, exosomes from transfected cells expressing one specific EBV component are likely to have a very different profile and biological effect compared with exosomes from virally infected cells. This is supported by our observations that EBER-containing exosomes from transfected cells appear to cause cell proliferation, whilst exosomes from virally infected cells...
which also contain EBERs induce apoptosis. It is also possible that the virus at different stages of its life cycle may induce the secretion of different viral and/or cellular components having very different or even opposite biological effects (Gutzeit et al., 2014; Klinker et al., 2014; Meckes, 2015). Moreover, the methodologies used for the isolation of exosomes and characterization of their contents can also lead to variability in the findings (Greening et al., 2015; Witwer et al., 2013).

In conclusion, our results indicate that exosomes from EBV-infected cells, but not non-infected cells, induce apoptosis in recipient cells via the extrinsic pathway involving FasL. We hypothesize that the exosomal FasL binds to CD95 (FasL receptor) on recipient cells and triggers the recruitment of the adaptor protein FADD (Fas-associated death domain) and the activation of the death-executing caspase-8 (Krammer, 2000). This in turn could activate the downstream effector caspases, notably caspase-7 and -3, resulting in apoptosis. Although several different cell types were susceptible to EBV exosome-mediated apoptosis, this phenomenon was by no means universal. The Burkitt’s lymphoma-derived B-cell line BJAB was found to be resistant to apoptosis, but it is unclear why. Another important issue emerging from this study is: what protects the parent cells from auto-exosome-mediated apoptosis? One possibility is that they downregulate their Fas receptor (Shisler et al., 1997). It is also possible that virally infected cells express FLIP (FLICE-like inhibitory protein)-like molecules which block FasL-mediated formation of DISC (death-inducing signalling complex) and the activation of caspase-8 (Guasparri et al., 2004). Clearly, much more work is required to unravel the intricate and complex interactions of EBV exosomes with their microenvironment.

**METHODS**

**Cell lines and culture.** For the isolation of exosomes, the following established cell lines were used: BL30 and BL30-B95.8 cells [EBV-negative and its corresponding EBV-positive Burkitt’s lymphoma cell lines of B-cell origin (Dellis et al., 2011) (kind gift of Professor Martin Rowe, University of Birmingham, UK)], Namalwa cells [EBV-positive Burkitt’s lymphoma cell line, expressing EBV type I latency pattern (Nadkarni et al., 1969)], BJAB cells [EBV-negative Burkitt’s lymphoma cell line of B-cell origin (Menezes et al., 1975) (kind gift of Professor Martin Rowe, University of Birmingham, UK)], B95.8 and GKLCL ([in vitro EBV-immortalized marmoset (Miller & Lipman, 1973) and human (Ahmed et al., 2014) LCLs, expressing EBV type III latency pattern] and 293T-pHEBo-E1 and 293T-pHEBo [stably transfected 293T cells with EBER1 or empty plasmid, respectively; the two plasmids were a gift from Professor Paul Farrell, Imperial College, London, UK (Gregorovic et al., 2011)].

The effect of exosomes isolated from EBV-infected and non-infected cells was tested on the following cells: 293T cells [EBV-negative human embryonic kidney epithelial cell line (Rio et al., 1985)], Jurkat cells [EBV-negative T-cell line (Schneider et al., 1977)], BL30 cells [EBV-negative B-cell line (Dellis et al., 2011)] and BJAB cells [EBV-negative B-cell line (Menezes et al., 1975)].

BL30, BL30-B95.8, Namalwa and Jurkat cells were grown in RPMI 1640 (Gibco) supplemented with 10 % FBS (Gibco), 1 % antibiotic/antimycotic solution (Santa Cruz), 50 µg gentamicin ml⁻¹ (HyClone) and 1 × glutamine (Gibco). Additional supplements for growth of BL30 and BL30-B95.8 were added in the media as described previously (Ahmed et al., 2014). 293T cells stably transfected with EBER1 plasmid (pHEBo-E1) or empty plasmid (pHEBo) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % FBS (Gibco), 1 % antibiotic/antimycotic solution (Santa Cruz), 50 µg gentamicin ml⁻¹ (HyClone) and 150 µg hygromycin B ml⁻¹ (Invitrogen). For exosome isolation, all cell lines were grown in media supplemented with 10 % exosome-depleted FBS (Eldh et al., 2012).

**Isolation of exosomes.** Exosomes were purified from culture supernatants of EBV-positive, EBV-negative and EBER1-transfected cell lines using differential ultracentrifugation as described previously (Ahmed et al., 2014). Briefly, for each cell line (cell density 0.5 × 10⁶ cells ml⁻¹ and viability > 95 %), 80 ml culture supernatant containing exosomes was centrifuged at 2000 g for 20 min at 4 °C to remove cells and cell debris. The supernatant was then centrifuged at 10 000 g for 30 min using a SW32 Ti rotor (Beckman) at 4 °C to remove any remaining cell debris. Exosomes were pelleted by ultracentrifugation at 100 000 g for 70 min at 4 °C. The exosome pellet was washed in PBS and ultracentrifuged at 100 000 g at 4 °C for 70 min. The final exosome pellet was resuspended in 100 µl PBS and the total exosome protein concentration was determined by the Bradford protein assay using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad).

**Exosome uptake assay.** Purified exosomes were labelled with PKH67 dye (Sigma) following the manufacturer’s instructions and as described previously (Thery et al., 2006). Briefly, 1 µl PKH67 dye was diluted in 50 µl diluent C provided in the kit. Then, 20 µl diluted PKH67 was added to 100 µl isolated exosomes and incubated at room temperature for 3 min. BSA/PBS (0.1 %) was then added and ultracentrifuged at 70 000 g for 60 min. The supernatant was carefully removed, and the exosome pellet was resuspended in 100 µl PBS and stored at −80 °C until required. For the exosome uptake assay, 293T cells (1 × 10⁴) were seeded onto glass coverslips and allowed to settle by incubating for 24 h at 37 °C in 5 % CO₂. On the next day, the cells were washed with 1 × PBS, and then 12.5 µg PKH67-labelled exosomes were added to the cells in exosome-depleted media and incubated for 24 h at 37 °C in 5 % CO₂. Following a 1 × PBS wash, cells were fixed in 4 % paraformaldehyde at room temperature for 30 min and counterstained with DAPI (Sigma). An Olympus DP71 fluorescence microscope was used for cell visualization.

**TEM.** Purified exosomes were visualized using TEM as described previously (Ahmed et al., 2014). Briefly, 10 µl exosome suspension was dried onto a freshly glow discharged 200 mesh Formvar-carbon-coated copper grid (Ted Pella) and negatively stained with 2 % aqueous uranyl acetate. Exosomes were observed with a Philips CM10 transmission electron microscope (Philips). Images were captured with a side-mounted 1K AMT Advantage digital camera (Advanced Microscopy Techniques).

**Western blotting on exosomes for CD63, flotillin, LMP1 and FasL.** The identity of exosomes seen under TEM was further confirmed by Western blotting for two well-known exosomal markers, i.e. CD63 (Verweij et al., 2011) and flotillin (Okabayashi & Kimura, 2010). CD63 Western blotting was performed using anti-CD63 mAb (Abcam) under non-reducing conditions and for flotillin using anti-flotillin mAb (Abcam) under reducing conditions, as recommended by the manufacturer. For the detection of FasL and LMP1 in exosomes, Western blotting was performed using anti-Fasl mAb (Abcam) and anti-LMP1 mAb (clones C51–4; Abcam), respectively. For all Western blots, 25–50 µg exosomal protein was loaded.

**Cell viability and caspase activation assays.** For the adherent cell line 293T, 2.5 × 10³ cells were incubated in 100 µl DMEM/10 % FBS
in 96-well flat-bottomed plates in duplicates or triplicates overnight. Normal media was then replaced with 100 µl exosome-depleted media and 12.5 µg exosomes. PBS was added in the negative control wells and cells were incubated for 48 h at 37 °C in 5 % CO2. For BL30 and Jurkat cell lines, 5 × 10^5 cells were incubated in 100 µl exosome-depleted RPMI/10 % FBS and 12.5 µg exosomes. PBS was added in the control wells and cells were incubated for 48 h at 37 °C in 5 % CO2. Cell viability was determined using a Cell Titre-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer’s instruction. The plates were read using a Perkin Elmer 2030 multilabel reader (Victor Tm X3) with the luminescence settings. To determine the activity of caspase-3/7, -8 and -9, the appropriate Promega Caspase-Glo kits were used. All assays were carried out in duplicates or triplicates and each experiment was independently repeated three times.

Caspase inhibition and FasL inhibition assays. For caspase-3/7/8 inhibition assays, caspase inhibitor II (CAS 210344-95-9; Calbiochem) was used. Cells were incubated with the inhibitor (25 mM final concentration) for 30 min prior to the addition of 12.5 µg exosomes. For FasL inhibition assay, anti-Fasl. antibody (Abcam) was added to 50 µl exosomes at a concentration of 0–100 µg ml^-1 and incubated for 1 h prior to using them for inducing apoptosis of target cells. Based on the results of this preliminary experiment, anti-Fasl. antibody at 50 µg ml^-1 was subsequently used with 50 µl exosomes and incubated for 1 h prior to using them for apoptosis assays. All assays were carried out in duplicates or triplicates and experiments repeated two or three times.

Soft agar colony formation assays. The soft agar assay was performed in six-well plates. An aliquot of 1 ml 2.4 % Bacto agar was added in each well and kept at 4 °C for 5–10 min. The base layer was then overlaid with a second layer consisting of 2.5 ml growth medium, 0.3 % Bacto agar and 3 × 10^5 293T cells, and left to set for 1 h. Then, 2 ml growth medium was added on top of the second layer. Exosomes (50 µg) were added immediately in test wells and PBS added to negative controls. Plates were incubated at 37 °C in 5 % CO2 for 5–14 days. Plates were fed twice a week with exosome-depleted media. The size of the colonies was observed between 5 and 14 days of treatment.

Statistical analysis. The data were analysed using the online OpenEpí software (http://www.openepi.com). The differences in the means of two groups were assessed by the independent sample t-test using two-tailed testing. A value of P<0.05 was considered to be statistically significant.

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REFERENCES


