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Exosomes Containing Glycoprotein 350 Released by EBV-Transformed B Cells Selectively Target B Cells through CD21 and Block EBV Infection In Vitro

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Exosomes are nano-sized membrane vesicles released from a wide variety of cells, formed in endosomes by inward budding of the endosomal limiting membrane. They have immune stimulatory-, inhibitory-, or tolerance-inducing effects, depending on their cellular origin, which is why they are used for use in vaccine and immune therapeutic strategies. In this study, we explored whether exosomes of different origins and functions can selectively target different immune cells in human peripheral blood. Flow cytometry, confocal laser scanning microscopy, and multispectral imaging flow cytometry (ImageStream) revealed that exosomes derived from human monocyte-derived dendritic cells and breast milk preferentially associated with monocytes. In contrast, exosomes from an EBV-transformed B cell line (LCL1) preferentially targeted B cells. This was not observed for an EBV+ B cell line (BJAB). Electron microscopy, size-distribution analysis (NanoSight), and a cord blood transformation assay excluded the presence of virions in our LCL1 exosome preparations. The interaction between LCL1-derived exosomes and peripheral blood B cells could be blocked efficiently by anti-CD21 or anti-gp350, indicating an interaction between CD21 on B cells and the EBV glycoprotein gp350 on exosomes. The targeting of LCL1-derived exosomes through gp350–CD21 interaction strongly inhibited EBV infection in B cells isolated from umbilical cord blood, suggesting a protective role for exosomes in regulating EBV infection. Our finding also suggests that exosome-based vaccines can be engineered for specific B cell targeting by inducing gp350 expression. The Journal of Immunology, 2011, 186: 000–000.

Exosomes (30–100 nm in size) were first described as waste products of maturing reticulocytes (1) and have since been found to be released from a wide variety of normal and malignant cells (2, 3). They have been isolated from biological fluids, including plasma and human breast milk (4, 5). Exosomes are formed by inward budding of the endosomal-limiting membrane (2, 6) and access the extracellular space when the endosome is emptied upon its fusion with the outer cell membrane. The composition of exosomes varies depending on their cell type of origin and their activation state. Exosomes from dendritic cells (DCs) express MHC class I and II molecules and are enriched in tetraspanins (e.g., CD63 and CD81), costimulatory molecules (2), and integrins and sphingolipids (7). They are considered key players in cell–cell communication because they can transfer Ag/MHC complexes (8–10) between cells. It was also shown that exosomes are involved in cell-to-cell transfer of mRNA and microRNA (11, 12), as well as infectious agents, including prions (13) and HIV (14). We previously reported that milk exosomes might be involved in tolerance induction (5), which was also observed for exosomes derived from intestinal epithelial cells (15). In contrast, B cell– (16, 17) and DC-derived exosomes (18) have mainly been implicated in T cell stimulation. However, a suppressive effect was recently demonstrated for exosomes derived from EBV-transformed B cell lines (LCLs) carrying functional viral microRNAs that repress EBV-targeted genes in noninfected bystander cells (12). The therapeutic potential of exosomes is being evaluated in clinical studies in which tumor peptide-loaded DC exosomes are used in cancer immunotherapy (19), and different types of exosomes are being investigated in vaccine strategies against various pathogens (13). However, fundamental mechanistic questions remain to be elucidated to understand the role of exosomes in vivo and the full potential of exosomes in clinical treatment.

In this study, we explored the nature of exosome-cell communication by investigating whether exosomes of various cellular origins with different functions target immune cells differently. Therefore, we compared the fate of tolerizing (breast milk), stimulatory (DCs), and repressive (LCL) exosomes with respect to their binding properties to immune cells in peripheral blood. Our results revealed that exosomes derived from human DCs and human breast milk preferably associated with monocytes, whereas exosomes from an LCL selectively targeted B cells. The molecule...
responsible for the exosome binding to B cells was found to be the EBV-derived glycoprotein gp350. The gp350-bearing exosomes efficiently blocked EBV infection in vitro. These findings suggest a role for exosomes in controlling EBV infection, as well as the possibility of developing exosome-based therapies in which B cell targeting is desired.

Materials and Methods
Exosome sources
Buffy coats from healthy blood donors at the blood bank of Karolinska University Hospital were used for Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) separation of PBMCs. Monocyte-derived DCs were generated as previously described (20), and their supernatants were collected between days 6 and 8. The EBV-transformed LCL1 cells, derived from a patient with birch pollen allergy, was a kind gift from Dr. Barbara Bohle (Medical University of Vienna, Vienna, Austria) (16). The EBV BJAB cell line (21) was a kind gift from Dr. Mikael Karlsson (Karolinska Institute). All cell lines were cultured in complete medium consisting of RPMI 1640 (Life Technologies, Invitrogen, Paisley, U.K.) supplemented with 25 mg/ml gentamicin (Life Technologies), 10% heat-inactivated and exosome-depleted FCS (Hyclone, Logan, UT), 2 mM/l t-glutamine, 100 IU/ml penicillin (Life Technologies), and 100 mg/ml streptomycin (Life Technologies). LCL1 cells were only used for up to 10 passages after they were thawed from a frozen stock. All cells were checked for mycoplasma infection using the MycoAlert mycoplasma detection kit (Lonza, Rockland, ME). Mature human breast milk was prepared as previously described (5) and stored at −80°C within 24 h of sampling. The study was approved by the local ethics committee; for milk collection, informed written consent was obtained from the donors.

Isolation of exosomes
Exosomes were isolated from cell-culture supernatants (DC or B cell lines) or from human cell-free milk by differential centrifugation, as previously described (5, 22). All three kinds of pelleted exosomes were resuspended immediately after the last wash in a small volume of PBS. If aggregates remained, solutions were filtered through a small-volume 0.2-μm syringe filter, before protein concentrations were determined using the Bio-Rad DC assay (Bio-Rad, Hercules, CA), according to the manufacturer’s instructions. The same amount of protein was used from each of the four exosome sources.

Staining of exosomes
Exosomes were stained with the green fluorescent membrane dye PKH67 (Sigma-Aldrich, St. Louis, MO). This was done by transferring exosomes from PBS to diluent C solution (Sigma-Aldrich) by centrifugation at 100,000 × g for 70 min. PKH67, diluted to 4 μM, and the exosomes (300 μg/ml) were filtered through small 0.2-μm syringe filters before mixing at 1:1 for 5 min, followed by the addition of 5% BSA and washing. Preliminary microscopic analysis showed that exosomes formed aggregates after staining (data not shown). To avoid these aggregates, the exosomes were resuspended in cell-culture media and filtered through a small-volume 0.2-μm syringe filter immediately before addition to cells. As a control, the same concentration of PKH67 was centrifuged in parallel to create a background control for possible pelleted unbound dye.

Coculture of PKH67-stained exosomes with PBMCs
Prefiltered PKH67-stained exosomes were added to PBMCs for 1 and 4 h at 37°C, as well as 4°C during temperature studies. Ten micrograms of exosomes per 5 × 10^5 PBMCs was added. The PKH67 dye pellet centrifuged in parallel was used as a background control.

Transmission and immune electron microscopy
Exosomes, which were precaptured on anti-QLA class II magnetic beads (23), and B cells cocultured with and without exosomes for 4 h were analyzed by transmission electron microscopy (TEM) as previously described (5). Exosomes without beads, the 10,000 × g pellet from LCL1 supernatants, and centrifuged (16,000 × g) supernatants from the EBV-producing B95-8 B cell line were processed by negative staining; an aliquot of 3 μl was added to a copper grid with a carbon-coated supporting film. The exosomes were stabilized by 0.5% uranyl acetate in water for 10 s, dipped in distilled water, and air-dried. Samples were examined using a Tecnai 10 transmission electron microscope (Fei, Acht, The Netherlands) at 80 kV.

Digital images were captured by a Mega View III digital camera (Soft Imaging System, Münster, Germany).

Flow-cytometry analysis
PBMCs were incubated with PKH67-stained exosomes and subsequently stained using two mouse mAb panels: 1) “allophycocyanin” anti–HLA-DR (MHC class II) PE-Cy5, anti–CD14-PE, and anti–CD19 Pacific Blue (or PE-Texas Red) and 2) “T cells” anti–CD8 allophycocyanin (or PE-Cy5), anti–CD4 PE, and CD3 Pacific Blue (BD Biosciences, San Jose, CA). The samples were run on a FACS Aria flow cytometer and analyzed by FACS Diva software (BD Biosciences). Gating was done first on lymphocyte/monocytes in forward light scatter/side scatter. For the allophycocyanin panel, the HLA-DR^-CD14^ and HLA-DR^-CD14^ populations were gated first. Thereafter, the CD19^- cells were gated from the HLA-DR^-CD14^- cells. For the T cell panel, the populations were selected as CD3^+CD4^- versus CD3^+CD4^ directly out of the lymphocyte/monocyte gate.

Confocal laser-scanning microscopy
PBMCs were incubated with PKH67-stained exosomes, centrifuged onto object slides (Shandon Cytopsin3, Block Scientific, Bohemia, NY), and fixed in 4% formaldehyde for 15 min. Staining was carried out using mAbs to CD3, CD14, or CD19 (BD Biosciences), according to the manufacturer’s instructions. A secondary goat anti-mouse mAb labeled with Alexa Fluor 546 (Molecular Probes, Eugene, OR) was used for detection. After staining, slides were mounted with 90% glycerol. Fluorescent images were acquired on a confocal laser-scanning microscope (TCS SP2, Leica Microsystems, Mannheim, Germany).

ImageStream analysis
Exosomes were stained and coincubated with PBMCs, as described for flow cytometry. Cells were run on an ImageStream X Mark IIA image-based flow cytometer, and images were analyzed using IDEAS image-analysis software (Amnis). Ten thousand events were collected in each sample, and single-stained compensation controls were used to compensate fluorescence between channel images on a pixel-by-pixel basis. Gating was done according to the principle for FACS. The cellular location of the PKH67 fluorescence was measured using the Internalization feature. The Internalization feature is defined as a ratio of the intensity inside the cell/intensity of the entire cell. The higher the score, the greater the concentration of intensity inside the cell. The inside of the cell is defined by an erosion of a mask that fits the membrane of the cell. The feature is invariant to cell size and can accommodate bright regions and small dim spots. The ratio is mapped to a log scale to increase the dynamic range to values between [−inf, inf]. Cells with primarily internal fluorescence have positive scores, whereas cells showing little internalization have negative scores.

Blocking assays
PBMCs (10^6/ml) were coincubated with mAbs to CD18 (clone MEM48), CD21 (clone B-E5), or isotype-matched Abs (20 μg/ml; Nordic BioSite, Taby, Sweden) for 30 min in cell-culture media at room temperature (RT); they were washed with PBS before exosomes were added at 10 μg/5 × 10^5 cells at 37°C for 1 or 4 h. FACS analysis was performed as previously described. LCL1 exosomes were treated with anti-CD23 (clone 9P25, 30 μg/ml; Beckman Coulter, Bromma, Sweden) or with the supernatant (30% of total volume) from a mouse hybridoma culture producing the gp350/250-neutralizing mAb 7A21 (DSMZ, Braunschweig, Germany). An irrelevant isotype control or hybridoma supernatant was used as control. After 30 min in cell-culture media at RT, pretreated LCL1 exosomes were added to B cells (10 μg/5 × 10^5 cells) for 4 h, before performing FACS analysis. B cells had been isolated from PBMCs using a B cell isolation kit II (Miltenyi Biotech/Fisher Scientific, Göttingen, Sweden).

Intracellular flow-cytometry staining
LCL1 and BJAB cells (5 × 10^6) were fixed with 4% formaldehyde for 5 min at RT. After three washes with PBS, cells were incubated for 10 min in 1% saponin (Sigma-Aldrich, Stockholm, Sweden) solution at RT. Cells were stained with the primary mAb 7A21 against gp350/250 by adding the supernatant from the mouse hybridoma culture at RT for 1 h. After washing twice with 0.1% saponin solution, cells were incubated with the secondary Alexa Fluor 488 (Invitrogen, Taastrup, Denmark) Ab for 45 min at RT, washed, and analyzed by flow cytometry.

Sucrose gradient
Fractions of exosome preparations were collected by sucrose gradient as previously described (5). Fractions were directly loaded onto anti-MHC
class II Dynabeads (Dynal, Oslo, Norway) for flow-cytometry analysis or centrifuged at 200,000 × g for 35 min at 4°C for immunoblot analysis.

**Immunoblot analysis**

Each pelleted exosome fraction was separated by SDS-PAGE (12%) and transferred to polyvinylidene difluoride membranes (Millipore, Solna, Sweden). Membranes were stained with mAbs to latent membrane protein 1 (LMP1) (clone CS. 1.4; DakoCytmation, Stockholm, Sweden), gp350 (clone 2L10; Millipore), CD81 (clone H-121; Santa Cruz Biotechnology, Heidelberg, Germany), or HLA-DR (clone TAL.1B3; DakoCytmation), according to the manufacturer’s instructions. Membranes were visualized with ECL Advance Western blotting Detection kit and exposed on Hyperfilms (GE Healthcare, Uppsala, Sweden).

**Cord blood transformation assay**

Heparinized cord blood samples, obtained from the Karolinska University Hospital and approved by the local ethics committee, were subjected to Ficoll-Paque density centrifugation. One million cord blood mono-nuclear cells (CBMCs) were incubated with the 10,000 × g pellet from BJAB or LCL1 supernatants or with 24 μg BJAB or LCL1 exosome preparations for 1.5 h in a humidified 37°C, 5% CO2 incubator. CBMCs were washed and resuspended in complete RPMI 1640 at 105 cells/ml and seeded in quintuplicate, at 2 × 105 cells per well/200 μL in 96-well plates. As a positive control for EBV-induced cell transformation, CBMCs were exposed to EBV (B95-8 virus)-containing supernatant. Culture medium served as negative control. CBMCs were fed weekly with fresh medium. On day 33, the transformation was registered visually by the appearance of typical cell aggregates and by thymidine-incorporation assay. One microCurie of [3H]thymidine (GE Healthcare, Uppsala, Sweden) was added to the cultures and incubated for 16 h. CBMCs were harvested onto glass fiber filters, and radioactivity was measured in a scintillation counter (1205 Beta-plate, PerkinElmer, Upplands Väsby, Sweden).

**NanoSight**

Size distribution within exosome preparations was analyzed by measuring the rate of Brownian motion using a NanoSight LM10 system, which is equipped with a fast video capture and particle-tracking software (NanoSight, Amesbury, U.K.) (24). EBV (strain B95-8) was used as a control. Prior to analysis with NanoSight, EBV was heat inactivated for 20 min at 56°C.

**EBV-infection assay/EBV-encoded nuclear Ag 2 induction assay**

B cells were isolated from human umbilical cord blood (approved by the local ethics committee) using a B cell isolation kit II (Miltenyi Biotec). A total of 5 × 105 B cells were left untreated or were preincubated for 2 h at 37°C with 100 μg BJAB or LCL1 exosomes. Thereafter, cells were infected with EBV by incubating them for 1 h at 37°C with B95-8 virus-containing supernatant. Cells were washed once with medium and centrifuged at 300 × g for 10 min. B cells were seeded onto a 48-well plate and cultured in 1 ml complete medium. Three days postinfection, cells were harvested and washed once in PBS, and the cell pellet was stored at −20°C. Cell pellets were lysed in loading buffer (16% glycerol, 100 mM Tris-HCl [pH 6.8], 3% SDS, 0.1% bromophenol blue, 4% 2-ME) and incubated for 10 min at 100°C, and proteins were separated through SDS-PAGE and analyzed by immunoblot for the expression of EBV-encoded nuclear Ag 2 (EBNA2; clone PE2, 1/5,000; Novocastra Laboratories, Newcastle, U.K.) and β-actin (clone AC-15, 1/400,000; Sigma-Aldrich). Polyclonal goat anti-mouse HRP (1/2000; DakoCytmation) was used as secondary Ab. Membranes were visualized with ECL Plus solution (GE Healthcare) and exposed on Hyperfilms (GE Healthcare).

**Statistical analysis**

The Wilcoxon matched-pairs test was applied using GraphPad Prism software, version 4.03 (Graphpad Software, San Diego, CA; http://www.graphpad.com); p values <0.05 were considered significant.

**Results**

**Exosomes derived from human DC and breast milk target monocytes, whereas LCL1 exosomes prefer B cells**

To elucidate the binding of exosomes to immune cells, we compared exosomes isolated from human monocyte-derived DCs, from an EBV-transformed lymphoblastoid B cell line (LCL1), and from human breast milk with regard to their adherence to PBMCs. Exosomes were stained with a green fluorescent membrane dye (PKH67) to be detectable by flow cytometry. To determine whether exosomes were properly labeled and whether their structures were retained after staining with PKH67, the exosomes were bound to magnetic anti-MHC class II beads (23). Flow-cytometry analysis showed that green fluorescent MHC class II-containing vesicles had been captured by the beads (Supplemental Fig. 1A), and TEM displayed nanovesicles with intact lipid bilayers, indicating that the PKH67 labeling did not interfere with exosome morphology (Supplemental Fig. 1B). The different exosomes were cocultured with PBMCs for 1 or 4 h and analyzed by multicolor flow cytometry to evaluate the association pattern of exosomes with cells. After 1 h, DC-derived exosomes mainly interacted with monocytes (HLA-DR^CD14^; average 46%), whereas only 17% bound to B cells (HLA-DR^CD14^/CD19^; Fig. 1A). In contrast, LCL1 exosomes showed the reverse pattern of association, with a strong preference for B cells. Sixty-three percent of the B cells were positive for LCL1 exosomes, whereas, on average, only 17% of the monocytes had associated with these exosomes at 1 h (Fig. 1B). After 4 h of coincubation there was a general increase in the percentages of exosome^+^ cells within each cell population, but the distinct association patterns with the different cell populations remained (Fig. 1D–F). Milk exosome interactions were generally low after 1 h of coincubation (Fig. 1C). However, at 4 h, 55% of the monocytes and 18% of the B cells had associated with milk exosomes (Fig. 1C), resembling the pattern for DC exosomes at 1 h. Previously, we found that milk exosome preparations have a lower content of exosomal vesicles in relation to the total protein amount in the exosome pellet compared with pellets from other exosome types (5). Accordingly, when increasing the amount of milk exosomes 5-fold (50 μg/5 × 105 PBMCs), the level of milk exosome interaction at 1 h reached an average of 64% with monocytes and 26% with B cells (n = 3; data not shown). These levels are comparable to those seen at 1 h when incubating with DC exosomes at 10 μg/5 × 105 PBMCs, suggesting higher amounts of nonexosomal proteins in the milk exosome preparations compared with the other exosome preparations.

Although T cells constitute the majority of PBMCs (~70% in this study), <8% of CD4^+^ or CD8^+^ T cells showed association with any of the exosomes after 4 h (Fig. 1). No consistent differences in the preferences for CD4^+^ compared with CD8^+^ T cells with the different exosome types were observed.

Exosomes are mainly associated with the cell membrane of B cells but are internalized by monocytes

Next, we asked where the exosomes localize within the different cell types. We coincubated PKH67-labeled exosomes with PBMCs and analyzed exosome association by confocal laser-scanning microscopy (CLSM). At 1 h, in general, no or only weak exosome signals could be detected in association with cells (data not shown), probably as a result of the lower detection level with CLSM compared with flow cytometry. After 4 h, DC exosomes were mainly internalized by monocytes (CD14^+) and, to a lesser degree, by B cells (CD19^+^), which often had cell membrane-associated exosomes (Fig. 2A). In contrast, LCL1 exosomes interacted to a higher degree with B cells and were mainly localized to the cell membrane (Fig. 2B). Monocytes showed weaker signals for LCL1 B cell exosomes (Fig. 2B). In general, milk exosomes showed weak signals, as seen in flow cytometry, and were detected as internalized in monocytes or associated with the cell membrane of B cells (Fig. 2C). In the few cases in which an interaction between exosomes and CD3^+^ T cells occurred (~1 of 50 cells), the exosomes were mainly localized near or in contact with the cell membrane (data not shown). Thus, these CLSM data are consistent with our flow-
cytometry data, showing that exosomes from EBV-transformed B cells preferentially target B cells, whereas DC-derived exosomes associate more with monocytes. However, these results also suggest that exosomes interact differently with different cell types; they are mostly associated with the cell membrane of B cells and T cells but are internalized by monocytes.

To verify the association and localization of different exosomes with immune cells by another technical approach, we decided to explore the ImageStream system. This system is a combined flow cytometer and fluorescence microscope that automatically captures events per sample in four-color flow cytometry. Data are expressed as the percentage of PKH67+ cells in each subpopulation. Horizontal lines represent mean values. Different blood donors are indicated by individual symbols.

The binding between LCL1-derived exosomes and B cells is temperature independent

To investigate whether the association of different exosomes with PBMCs is receptor mediated or dependent on active internalization, we cocultured PKH67+ exosomes with PBMCs at 4 and 37˚C. For all three exosome types, the association with monocytes decreased when incubation was performed at 4˚C for 1 and 4 h (Fig. 3), indicative of an active, probably phagocytic, uptake by this cell type. DC (Fig. 3A, 3D) and milk exosome (Fig. 3C, 3F) associations with B cells were similarly diminished during cold conditions. In contrast, only a slight decrease in interaction between LCL1 exosomes and B cells was seen at 4˚C at both time points (Fig. 3B, 3E). Thus, the interaction between LCL1 exosomes and B cells was largely independent of temperature; therefore, it is more likely mediated through adhesion molecules or surface receptors, which is why we set out to dissect this interaction further.

The binding between LCL1-derived exosomes and B cells is dependent on CD21 expressed on the B cells

B cells express cell-surface receptors, such as the human complement receptor 2 (CD21), which, together with a distinct pattern of adhesion molecules [e.g., ICAM-1 or integrins, such as LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), or p150,95 (CD11c/CD18) (17)], may mediate the observed strong B cell preference of B cell exosomes. To reveal the specificity of the exosome binding, mAbs against CD21 or CD18 were added to PBMCs before incubation with exosomes. The interaction between LCL1 exosomes and peripheral blood B cells was blocked efficiently by anti-CD21, whereas no blocking effect was observed with anti-CD18 Abs (Fig. 4A).

To elucidate whether the B cell targeting observed was dependent on the EBV transformation of the B cells, we compared it with exosomes from the EBV− B cell line BJAB (22). Results showed that BJAB exosomes bound to B cells 10 times less than LCL1 exosomes, suggesting the involvement of EBV-derived or -induced proteins in the binding (Fig. 4B). Taken together, these data indicate that the binding between LCL1 exosomes and B cells is dependent on an interaction with CD21 and not on LFA-1, Mac-1, or p150,95, and suggest a selective B cell exosome targeting, which was specific for the exosomes derived from the EBV-transformed B cells.

gp350 on LCL1 exosomes mediates the binding to B cells

Next, we aimed to elucidate the exosomal ligand involved in the CD21 binding on B cells. Known ligands to CD21 include the low-affinity receptor for IgE (CD23), the EBV envelope glycoprotein gp350, and the complement factor C3d (25). It was shown that exosomes released from B cells and macrophages contain C3 fragments (26), but because heat-inactivated FCS was used in all of our cell cultures, C3d is unlikely to make a difference. CD23, which is highly expressed on EBV-transformed B cell lines (27), was detected on our LCL1 exosomes but not on BJAB exosomes (Supplemental Fig. 2). The EBV glycoprotein gp350 is a structural protein that is critical for viral attachment to B cells (28), but it has not been shown to be present on exosomes. Hence, gp350 and CD23 were the first candidates to be investigated for their possible involvement in the exosome–B cell interaction. These ligands were blocked by anti-gp350/220 (29) or anti-CD23 Abs (25). The
binding of LCL1 exosomes to B cells was substantially reduced when gp350 was blocked; interestingly, however, no reduction in exosome binding was seen when CD23 was blocked (Fig. 4C). This observation suggests the presence of gp350 on the surface of LCL1 exosomes and that this EBV glycoprotein, and not CD23, mediates the exosome binding. As a control, we also added a nonneutralizing anti-gp350 mAb (2L10) (29), which did not block exosome binding (data not shown), reinforcing the notion of a specific blocking of gp350 via the neutralizing mAb (72A1).

Herpesvirus-infected cells express viral glycoproteins on their cell surface during the lytic phase of the viral life cycle. Therefore, we investigated the presence of gp350 on LCL1 cells by flow cytometry (Fig. 4D). Interestingly, we observed that a high proportion of LCL1 cells expressed gp350 on the cell surface, as well as intracellularly. To verify the presence of gp350 on LCL1 exosomes, exosomes were purified through sucrose gradient, and fractions were analyzed by immunoblotting (Fig. 4E). Raposo et al. (22) demonstrated that LCL-derived exosomes float in a sucrose gradient at a density of 1.08–1.22 g/ml. Immunoblot analysis for exosome-enriched HLA-DR and CD81 within a density of 1.10–1.19 g/ml for LCL1 and BJAB exosome preparations verified the presence of exosomes. LMP1 was found on exosomes secreted by LCLs (30), and we also demonstrated its presence on the LCL1 exosomes. Intriguingly, gp350 was also detected on LCL1 exosomes. However, gp350 expression on LCL1 exosomes did not fully overlap with LMP1 expression, suggesting a heterogeneous exosome population. As expected, neither gp350 nor LMP1 was detected on BJAB exosomes (Fig. 4E).

**Exosome signals are derived from exosomes and not virions**

The expression of gp350 on LCL1 cells and exosomes raises the question of whether some of our LCL1 cells are in a lytic stage of the EBV life cycle, which would suggest that EBV may also reside in the EBV-transformed B cell cultures as free virus particles. Therefore, there is a possibility that virions might contaminate our exosome preparations and, thereby, give a false-positive signal for exosomes in our experiments. In preliminary experiments, we detected viral DNA by PCR; however, the presence of DNA does not always correspond to the presence of complete virions. Hence, the more sensitive cord blood-transformation assay was performed to investigate the presence of infectious EBV, as well as TEM analysis to detect EBV particles in our exosome preparations. The 10,000 × g pellets (obtained during an intermediate centrifugation step during the exosome-isolation procedure), as well as exosome preparations from BJAB and LCL1 supernatants, were added to CBMCs to monitor the outgrowth of EBV-transformed primary B cells (Fig. 5A). Infection with the B95-8 virus served as a positive control. The addition of pooled 10,000 × g pellets from BJAB supernatants did not induce the outgrowth of LCLs, as quantified by [3H]thymidine incorporation. In addition, visual examination of the cultures did not reveal any typical aggregates of transfected B cells. In contrast, the addition of pooled 10,000 × g pellets from LCL1 supernatants to the CBMCs induced the outgrowth of LCLs, indicating the production of infectious virus particles by LCL1 cells. This finding is in line with the observed cell-surface expression of gp350 on LCL1 cells (Fig. 4D).
indicating that LCL1 cells are in a lytic, virus-producing stage. However, no outgrowth of LCLs was observed after the addition of exosome preparations (100,000 $g$) from BJAB and LCL1 supernatants to CBMCs, indicating the absence of infectious EBV within our LCL1 exosome preparations. Furthermore, we performed TEM analysis of the 10,000 $g$ pellet (Fig. 5B) and the 100,000 $g$ exosome pellet (Fig. 5C) from LCL1 supernatants and compared the morphology of the observed particles and vesicles with TEM photographs of virions from supernatants of B95-8 cells (Fig. 5D). TEM analysis of the 10,000 $g$ pellet confirmed the presence of viral particles similar to those observed in B95-8 supernatants and as demonstrated for another virus-producing cell line (31). In contrast, no viral particles were detected in the 100,000 $g$ exosome pellet from LCL1 supernatants (Fig. 5C).

FIGURE 3. The binding between LCL1-derived exosomes and B cells is independent of temperature. PBMCs were incubated with PKH67-labeled exosomes from DCs (A, D), EBV-transformed B cells (LCL1) (B, E), or breast milk (C, F) for 1 or 4 h at 4 or 37˚C and analyzed as described in Fig. 1. Data are percentages of exosome$^+$ cells within each subpopulation of HLA-DR$^+$ cells measured as PKH67$^+$ by flow cytometry. Mean ± SD for PBMCs from three donors are shown.

FIGURE 4. The interaction between B cells and exosomes derived from EBV-transformed B cells is mediated by interactions between CD21 and gp350. PBMC cultures were treated with anti-CD21, anti-CD18, or isotopic-matched controls (20 μg/ml), before LCL1 exosomes (A) or BJAB exosomes (B) were added for 4 h. C, LCL1 exosomes were preincubated with anti-gp350 supernatant from the 72A1 mouse hybridoma (30% of total volume), anti-CD23 (30 μg/ml), or isotypic-matched controls and then added to purified B cells for 4 h. Representative flow-cytometry dot plots (right panels), including percentage numbers, show LCL1 exosome association with B cells treated with isotypic control (upper panel) or anti-gp350 mAbs (lower panel). Analysis was performed as described in Fig. 1. Mean ± SD for PBMCs from three donors are shown (A–C). D, Flow-cytometry graphs show extracellular (left panel) and intracellular (right panel) expression of gp350, comparing BJAB cells with LCL1 cells. Ten thousand cells were analyzed. E, Pellets of sucrose gradient fractions from LCL1 (left panel) and BJAB (right panel) exosome preparations were analyzed by immunoblot using Abs against LMP1, gp350, CD81, and HLA-DR. The density of each fraction was determined by refraction index measurements. One representative experiment of three is shown.
exosomes by immunogold labeling for CD63 and HLA-DR (Fig. 5F). Exosomes attached to primary B cells (Fig. 5G) or exosome preparations alone (Fig. 5C, 5F) did not reveal any EBV particles in the size of infectious EBV (200 nm) or naked high-density virus capsids.

gp350-containing LCL1 exosomes reduce EBV infection of primary B cells

To determine the functional consequences of gp350 exosome binding to B cells, we investigated whether LCL1 exosome binding has an impact on EBV infection. Therefore, B cells isolated from umbilical cord blood were preincubated with BJAB or LCL1 exosomes or left untreated prior to EBV exposure. Successful EBV infection was monitored after 3 d by immunoblotting for expression of latent protein EBNA2. Intriguingly, preincubation of B cells with LCL1 exosomes strongly reduced subsequent EBV infection, because reduced EBNA2 protein levels were observed (Fig. 6). This strong inhibitory effect was not observed when B cells were preincubated with control BJAB exosomes; only a slight reduction in EBNA2 expression was seen.

Discussion

This study showed that exosomes from a LCL line that expresses the viral structural protein gp350 on the surface are capable of specifically targeting B cells. We also demonstrated that these exosomes can block EBV infection in vitro. This suggests a role for exosomes in the regulation of EBV infection.
EBV particles in our exosome preparations, which could be re-
raised the question whether we had infectious and/or pleiomorphic (data not shown).

higher extent compared with the exosomes from immature DCs
omes from LPS-activated DCs attached to monocytes to an even
pient cell, as well as the maturation status of the cell producing the
stimulated T cells. The activation state of the exosome-reci-
that we used ex vivo PBMCs, likely to contain mainly non-
pared with inactive T cells (37). Therefore, our low degree of
pression of LFA-1 attract DC exosomes to a higher degree com-
recently reported that activated T cells with an upregulated ex-
B cell exosomes are known to display the ICAM-1 that binds to
prior to EBV exposure. Three days postinfection, B cells were analyzed by
munoblot for EBNA2 and β-actin expression. Phase-contrast micro-
images of B cells preincubated with BJAB exosomes, LCL1 exo-
somes, or left untreated prior to subsequent EBV infection, and
noninfected B cells (from left to the right). One representative experiment
of three is shown. Original magnification ×5. Scale bar, 100 μm.

It is known that different cell types produce exosomes with
phenotypes that mainly reflect their cells of origin (32, 33). In this
study, we looked at the other side of the exosome communication
pathway and demonstrated that the exosomes tested, from
monocyte-derived DCs and breast milk, seem to have the same
main target in human peripheral blood (i.e., monocytes). Reaching
the monocytes, the exosomes seem to be actively engulfed,
probably by phagocytosis, which was also recently demonstrated
to be the mechanism of exosome uptake by other phagocytes, such
as macrophages (34). However, this paper also showed that the
selectivity of exosomes to target monocytes might change if the
exosome-producing cell carries pathogen-specific molecules,
which we demonstrated in this study by comparing exosomes
from an EBV− (BJAB) cell line with an EBV+ B cell line (LCL1).
LCL1-derived exosomes mainly targeted B cells; however,
this was not seen for BJAB exosomes. The interaction between
LCL1 exosomes and B cells was efficiently blocked by Abs to
CD21 on B cells or Abs to gp350 on exosomes but not by anti-
CD18 or anti-CD23, demonstrating the involvement of EBV.
The reason why anti-CD23 had no effect on the cell–exosome in-
teraction, although CD23 is more abundant on LCL1 compared
with BJAB (Supplemental Fig. 2), might be due to a lower affinity
interaction between CD23 and CD21 compared with the gp350–
CD21 interaction. This indicates that a very high molecular af-
finity (35) is needed for rapid (1 h) binding of exosomes in vitro
and might be even more important in vivo.

The interaction of the various exosomes with T cells was rather
low (<8% of T cells positive for exosomes), although DC and
B cell exosomes are known to display the ICAM-1 that binds to
LFA-1 expressed on, for example, T cells (17, 36). However, it was
recently reported that activated T cells with an upregulated ex-
pression of LFA-1 attract DC exosomes to a higher degree com-
pared with inactivated T cells (37). Therefore, our low degree of
exosome–T cell interaction (Fig. 1) might be explained by the fact
that we used ex vivo PBMCs, likely to contain mainly non-
stimulated T cells. The activation state of the exosome-recipient
cell, as well as the maturation status of the cell producing the
exosomes, is important. Our preliminary data showed that exo-
somes from LPS-activated DCs attached to monocytes to an even
higher extent compared with the exosomes from immature DCs
(data not shown).

The observation that gp350 was present on the LCL1 exosomes
raised the question whether we had infectious and/or pleiomorphic
EBV particles in our exosome preparations, which could be re-
ponsible for the PKH67 signal seen on B cells. The binding of EBV
to CD21 is well established (27). By using the sensitive cord blood-
transformation assay, as well as TEM analysis (Fig. 5A–D), we did
not find any evidence for virions in our exosome preparations.
Thus, exosome preparations were cleared of virions by centrifu-
gation; therefore, we consider it unlikely that virions were re-
sponsible for the fluorescence signal seen on B cells.

It should be mentioned that the LCL1 used in this study does not
represent a typical LCL, because their cells have a regular round
shape, and it produces virions. However, this is an advantage when
the aim is to study exosomes produced during the lytic phase of
EBV infection. Further investigations are warranted to virologi-
cally characterize and compare the LCL1 cell line with other LCLs
with respect to the expression of viral and cellular gene products,
which was already shown to vary in different LCLs (38).

The finding that binding of gp350 containing LCL1 exosomes to
B cells inhibited subsequent EBV infection (Fig. 6) suggests that
exosomes play a role during the early phases of EBV infection.
Thus, lytically infected B cells secrete gp350-containing exosomes
that bind to CD21 on bystander B cells, thereby protecting them
from subsequent EBV infection (39). This innate-like immune
mechanism might control EBV infection, in addition to the pow-
erful adaptive immune responses. We also observed a slight in-
hibition of EBV infection when B cells were preincubated with
BJAB exosomes. A possible explanation is that these exosomes
unspecifically bind to added EBV particles and, thereby, sterically
hinder them from infecting B cells.

In the current study, we also observed that the EBV-transformed
B cells seemed to produce more exosomes, measured as protein
concentration, compared with the EBV+ B cells (data not shown).
This may also mirror the situation in vivo, in which a high number
of exosomes may contribute to control the spread of EBV in-
festation. Alternatively, the induction of exosome production and
the exosomal expression of gp350 may contribute to the immu-
nomodulatory potential of EBV. This was recently suggested for
HSV-1, for which glycoprotein B is able to manipulate the class
II-processing pathway, whereby the protein hijacks HLA-DR from
its normal transport route and directs it into the exosomal pathway
(40). The recent finding of viral microRNA in LCL exosomes may
also indicate that exosomes are used to deliver viral messages
between cells (12). Further investigations are needed to under-
stand the extent to which exosomes are involved in the regulation
of EBV infection or spreading.

Our findings also suggest how exosomes can be engineered
(e.g., by inducing the expression of gp350) to redirect their cellular
targeting to B cells, which may potentiate their therapeutic use-
fulness. A role for B cells in producing a complete T cell response
was suggested in the 1980s (41). Later, Ding et al. (42) showed
that targeting of Ags to B cells can potentiate specific T cell
responses and break immune tolerance. Furthermore, B cells are
particularly important in achieving long-term T cell immunity
(43) and, recently, we showed that exosomes require the support
of activated B cells for generating Ag-specific T cell responses
in vivo (44). Hence, by targeting B cells in cancer vaccines,
tolerance could be broken, and a more long-lasting T cell im-
munity might be achieved. Furthermore, CD21 is expressed by
B cells, as well as by follicular DCs (45). This implies that
exosomes with surface-associated gp350 may also target follicular
DCs in vivo, thereby enhancing a possible immune activation and
memory in vivo.

The direct use of exosomes from EBV-transformed B cells is
a possibility; however this approach could have limitations in
a clinical setting because of the presence of LMP1. LMP1 is known
to inhibit T cell proliferation (30) and was reported to have onco-
genic properties (46). Still, several studies showed the potential of

![Figure 6](http://www.jimmunol.org/Downloaded_from.jpg)

**FIGURE 6.** gp350-containing LCL1 exosomes strongly reduce EBV infection of primary B cells. Umbilical cord blood B cells were pre-
incubated for 2 h with BJAB or LCL1 exosomes or were left untreated (−) prior to EBV exposure. Three days postinfection, B cells were analyzed by
munoblot for EBNA2 and β-actin expression. Phase-contrast microscopy images of B cells preincubated with BJAB exosomes, LCL1 exo-
somes, or left untreated prior to subsequent EBV infection, and
noninfected B cells (from left to the right). One representative experiment
of three is shown. Original magnification ×5. Scale bar, 100 μm.
exosomes, derived from EBV-transformed B cells, to activate T cells (16, 22). In addition, the relative expression of LMP1 and gp350 on exosomes may differ during the latent and lytic phase; therefore, the exosome cultures may be fine-tuned for optimal clinical applications. An alternative strategy would be to modify DC exosomes, which have been implicated in clinical studies (19), to express gp350.

In conclusion, we showed that exosomes, found in breast milk and produced by human monocyte-derived DCs and an EBV+ B cell line, do not preferably associate with B cells. Instead, they mainly target monocytes, which actively engulf exosomes, as demonstrated for milk and DC exosomes. However, if B cells harbor EBV in their lytic stage, the produced exosomes change their preference from monocytes toward B cells, whereby exosome-associated gp350 binds to the EBV entry receptor CD21 on B cells. The specific binding of these exosomes to B cells strongly reduced EBV infection and suggests a protective role for exosomes during EBV infection. Furthermore, exosomes targeting B cells might be efficient in inducing long-term cellular and humoral immune responses; hence, they should be considered potential tools in the treatment of cancer and inflammatory diseases.

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Disclosures

S.F. and T.C.G. are employees of Amnis Corporation, which manufactures ImageStream. S.G. has a patent pending on exosome-based treatment of cancer.

References


Supporting information

Exosomes containing gp350 released by Epstein-Barr virus transformed B cells selectively target B cells through CD21 and block EBV infection in vitro

Supplementary Figure S1. Exosomes from DC, EBV transformed B cells and breast milk are MHC class II positive and exosomes maintain their morphology after staining with PKH67. (A) Histogram with overlays showing PKH67+ exosomes from human breast milk (solid line), EBV transformed B cells (dashed line) and DC (dotted line) coated on anti-MHC class II beads and analyzed by flow cytometry. The filled histogram shows the anti-MHC class II beads with PKH67 without exosomes. PKH67 fluorescence was detected in the FITC channel and a minimum of $5 \times 10^3$ beads were analyzed per sample. (B) Transmission electron microscopy confirmed the morphology of the stained exosomes. B cell-derived exosomes are shown as a representative example from two experiments.
Supporting information

Exosomes containing gp350 released by Epstein-Barr virus transformed B cells selectively target B cells through CD21 and block EBV infection in vitro

Supplementary Figure S2. LCL1 and LCL1-derived exosomes express CD23. (A) Flow cytometry histograms show expression of CD23, comparing BJAB cells (grey) with LCL1 cells (black line). Results shown are from one representative experiment out of 3. (B) Expression of CD23 on exosomes derived from BJAB cells (as a grey background) and LCL1 cells (black line) was analyzed by flow cytometry using anti-MHC-class Dynabeads. Results shown are from one experiment, where ten thousand events were analyzed.