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

Helen Vallhov, Cindy Gutzeit, Sara M. Johansson, Noémi Nagy, Mandira Paul, Qin Li, Sherree Friend, Thaddeus C. George, Eva Klein, Annika Scheynius and Susanne Gabrielsson

*J Immunol* published online 24 November 2010
http://www.jimmunol.org/content/early/2010/11/23/jimmunol.1001145
Exosomes Containing Glycoprotein 350 Released by EBV-Transformed B Cells Selectively Target B Cells through CD21 and Block EBV Infection In Vitro

Helen Vallhov,*† Cindy Gutzeit,*† Sara M. Johansson,† Noémi Nagy,† Mandira Paul,* Qin Li,† Sherree Friend,‡ Thaddeus C. George,‡ Eva Klein,† Annika Scheynius,* and Susanne Gabrielsson*

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 investigated 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 preferably 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...
Materials and Methods

Exosome sources

 Buffy coats from healthy blood donors at the blood bank of Karolinska University Hospital were used for Ficoll-Paque (Amer sham 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 mmol/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, Rock- land, 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 protein 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-HLA 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. After 1 min of saturation, 1 μl of the exosome suspension was deposited on the grid with a 25-μl pipette tip. The sample was air-dried and then stained with 2% uranyl acetate in water for 10 s, followed by exposure to 50% formic acid and 2% ammonium acetate in water for 10 s, then washed distilled water, and air-dried. Samples were examined using a Tecnai 10 transmission electron microscope (FEI, Acht, The Netherlands) at 80 kV.

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 (both from 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 aconfocal 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 multispectral imaging 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 concentrated 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 72A1 (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/2.5 × 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, Göteborg, Sweden).

Intracellular flow-cytometry staining

LCL1 and BJAB cells (5 × 10^5) 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 72A1 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, Taestrup, 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; DakoCytomation, Stockholm, Sweden), gp350 (clone 2L10; Millipore), CD81 (clone H-12; Santa Cruz Biotechnology, Heidelberg, Germany), or HLA-DR (clone TAL.1B5; DakoCytomation), 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 10^5 cells/ml and seeded in quintuple, at 2 × 10^5 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 Betaplate, 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. 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.

**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 × 10^6 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 LS, 0.1%; DakoCytomation, Stockholm, Sweden), gp350 (clone 2L10; Millipore), CD81 (clone H-121; Santa Cruz Biotechnology, Heidelberg, Germany), or HLA-DR (clone TAL.1B5; DakoCytomation), according to the manufacturer's instructions. Membranes were visualized with ECL Advance Western blotting Detection kit and exposed on Hyperfilms (GE Healthcare, Uppsala, Sweden).

**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 coincubated 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 × 10^5 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 × 10^5 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 multispectral images of each cell that passes through the flow cell at 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).

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, Abs 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).
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 heterogenous 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 transformed 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). In addition, we compared the size distribution of exosome preparations from BJAB and LCL1 cells with EBV-containing B95-8 supernatants by nanoparticle tracking analysis (NanoSight). This method quantitatively confirmed the lower size range within the LCL1 and BJAB exosome preparations (average ~100 nm) compared with EBV, which had a size range of 150–200 nm (Fig. 5E), further supporting the absence of virions in our exosome preparations. Finally, we confirmed the presence of

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 isotype-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 isotype-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 isotype 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.
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). The 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 interaction, although CD23 is more abundant on LCL1 compared to 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 affinity (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 expression of LFA-1 attract DC exosomes to a higher degree compared with inactive 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 nonstimulated T cells. The activation state of the exosome-receptor cell, as well as the maturation status of the cell producing the exosomes, is important. Our preliminary data showed that exosomes 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 responsible 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 centrifugation; therefore, we consider it unlikely that virions were responsible 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 virologically 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 powerful adaptive immune responses. We also observed a slight inhibition 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 infection. Alternatively, the induction of exosome production and the exosomal expression of gp350 may contribute to the immunomodulatory 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 understand 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 usefulness. 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 immunity 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- genetic properties (46). Still, several studies showed the potential of
exosomes, derived from EV-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 EV 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.

Acknowledgments
We thank Prof. Ingemar Ernberg, Department of Microbiology, Tumour and Cell Biology, and Dr. Mikael Karlsson, Department of Medicine Solna, Karolinska Institutet, for helpful discussions. We also thank Dr. Kjell Haltenby and staff at the Electron Microscopy Unit, Karolinska University Hospital, Huddinge, Sweden, and Sven Petersson, School of Cancer Sciences, University of Birmingham, Birmingham, U.K., for excellent support with TEM. Prof. Peter Biberfeld, Department of Pathology and Tumor Biology, Karolinska Institutet, shared EBV TEM expertise. We also thank Dr. Andrew Malloy for analyzing the exosome preparations with NanoSight.

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

Downloaded from http://www.jimmunol.org/ by guest on April 22, 2017


