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Exosomes Derived from Burkitt’s Lymphoma Cell Lines Induce Proliferation, Differentiation, and Class-Switch Recombination in B Cells

Cindy Gutzeit,* Noemi Nagy,† Maurizio Gentile,‡ Katarina Lyberg,§ Janine Gumz,* Helen Vallhov,* Irene Puga,† Eva Klein,‡ Susanne Gabrielsson,* Andrea Cerutti,‡ and Annika Scheynius*

Exosomes, nano-sized membrane vesicles, are released by various cells and are found in many human body fluids. They are active players in intercellular communication and have immune-suppressive, immune-regulatory, and immune-stimulatory functions. EBV is a ubiquitous human herpesvirus that is associated with various lymphoid and epithelial malignancies. EBV infection of B cells in vitro induces the release of exosomes that harbor the viral latent membrane protein 1 (LMP1). LMP1 per se mimics CD40 signaling and induces proliferation of B lymphocytes and T cell–dependent class-switch recombination. Constitutive LMP1 signaling within B cells is blunted through the shedding of LMP1 via exosomes. In this study, we investigated the functional effect of exosomes derived from the DG75 Burkitt’s lymphoma cell line and its sublines (LMP1 transfected and EBV infected), with the hypothesis that they might mimic exosomes released during EBV-associated diseases. We show that exosomes released during primary EBV infection of B cells harbored LMP1, and similar levels were detected in exosomes from LMP1-transfected DG75 cells. DG75 exosomes efficiently bound to human B cells within PBMCs and were internalized by isolated B cells. In turn, this led to proliferation, induction of activation-induced cytidine deaminase, and the production of circle and germline transcripts for IgG1 in B cells. Finally, exosomes harboring LMP1 enhanced proliferation and drove B cell differentiation toward a plasma-blast-like phenotype. In conclusion, our results suggest that exosomes released from EBV-infected B cells have a stimulatory capacity and interfere with the fate of human B cells. *The Journal of Immunology, 2014, 192: 000–000.

Exosomes are nano-sized membrane vesicles (40–100 nm in diameter) that are formed by inward budding of the endosomal membrane within multivesicular bodies (1). Upon fusion of the multivesicular body membrane with the plasma membrane, exosomes are released into the environment where they can exert their function as immune mediators on bystander cells (2). Many cell types, including immune cells such as dendritic cells (DCs) and B and T cells, release exosomes, and they are found in human body fluids, such as plasma, saliva, urine, and breast milk (3). Cellular activation is needed to induce exosome release by primary immune cells, in particular primary B cells (4). The physiological role of exosomes remains to be fully elucidated, but many studies provide strong evidence that they are active players in intercellular communication as a result of their immune-suppressive, immune-regulatory, and immune-stimulatory functions (5–8).

EBV is a ubiquitous human γ herpesvirus that successfully coevolved with its host to persist in a latent stage within isotype-switched memory (IgD+CD27+) and nonswitched marginal zone (IgD-CD27+) B cells (9–11). It is the causative agent of infectious mononucleosis and is associated with lymphoid and epithelial malignancies, such as posttransplant lymphoproliferative disorders, Hodgkin’s disease, Burkitt’s lymphoma, and nasopharyngeal carcinoma (12). Intriguingly, EBV is also suspected to contribute to autoantibody production in patients suffering from autoimmune diseases, such as systemic lupus erythematosus, multiple sclerosis, and rheumatoid arthritis (13). In vitro EBV-transformed B cells (lymphoblastoid cell line [LCL]) constitutively release exosomes that induce Ag-specific MHC class II–restricted T cell responses (14). Moreover, exosomes released by LCLs harbor the EBV latent membrane protein 1 (LMP1) (15). LMP1 function mimics CD40 signaling and thereby ensures EBV persistence within the B cell compartment by promoting apoptotic resistance, proliferation, and immune modulation (16). LMP1 is constitutively active and signals in a ligand-independent fashion through mitogen-activated kinases, NF-κB, and the JAK/STAT pathway via TNFR-associated factors (17). Thus, LMP1 expression must be tightly regulated during EBV infection. Recently, it was demonstrated that constitutive LMP1 signaling within B cells is blunted through the shedding of LMP1 via exosomes (18).
Therefore, LMP1 exosomes released by infected cells during EBV-associated diseases might contribute to clinical features seen in patients with lymphoproliferative disorders or autoimmune diseases. Recombinant LMP1 was shown to directly suppress activated T cells, and exosomes released by EBV-infected nasopharyngeal carcinoma cells harbor LMP1 (19, 20). Both studies suggest that LMP1 secreted by EBV+ tumor cells might mediate immunosuppressive effects on tumor-infiltrating lymphocytes. However, a potential effect of LMP1 exosomes on B cells equipped with all CD40-signaling molecules has not been addressed.

In vivo administration of OVA-loaded DC-derived exosomes is able to induce Ag-specific CD4+ T cell responses through a B cell–dependent mechanism, suggesting exosomes as Ag shuttle systems for delivery to B cells (21). In this study, we examined whether B cell–derived exosomes are conveyers of intercellular communication by interfering with the fate of human B cells. To mimic exosomes released during EBV infection or EBV-associated diseases, we took advantage of the human EBV− DG75 Burkitt’s lymphoma cell line and its derived sublines (LMP1 transfected and EBV infected) as a stable source of human B cell–derived exosomes carrying LMP1 or not. We addressed their functional potency and tested the hypothesis of whether LMP1 transferred via exosomes exerts its function after binding and internalization by B cells. In this study, we demonstrate that exosomes harboring LMP1 were released during primary EBV infection of B cells and that similar physiological conditions were found on exosomes secreted from DG75-LMP1 cells. When exposed to DG75 exosomes, human peripheral B cells gained the capacity to proliferate, upregulated the expression of activation-induced cytidine deaminase (AID), and induced intronic γ1 exo–C region of the H chain μ(γ1-Clμ) circle and γ1/1/2-Cγ1 germline transcripts. Additionally, exosomes harboring LMP1 induced differentiation toward a plasmablast-like phenotype. Altogether, our study highlights the B cell–stimulatory capacity of exosomes released by EBV-infected B cells. We propose that clinical features observed in patients with EBV-associated diseases, such as lymphoproliferative disorders or autoimmune diseases, might be intensified by the presence and action of these exosomes.

Materials and Methods

B cell lines

The following B cell lines were used for exosome preparations: EBV− Burkitt’s lymphoma DG75-CO (control), DG75-LMP1 (stably transfected with LMP1), DG75-EBV (EBV infected) (22–24), BJAB, and lymphoblastoid cell line LCL1 (25). Cells were tested routinely and were mycoplasma free (VenvorGem; Minerva Biolabs); they were cultured (5 × 10^5) in RPMI 1640 medium supplemented with 10% FCS (HyClone, Nordic Biolab; FCS was diluted with RPMI 1640 medium supplemented with 2 mM L-glutamine (HyClone), 100 IU/ml penicillin and 100 mg/ml streptomycin (HyClone), 2 mM L-glutamine (HyClone), 100 IU/ml penicillin and 100 mg/ml streptomycin (HyClone), and 1 mM sodium pyruvate (Sigma-Aldrich) at 37°C, 5% CO2. After 3 d, the culture supernatants were collected for exosome isolation.

Exosome isolation and phenotyping

B cell–derived exosomes (DG75-Coex, DG75-LMP1ex, DG75-EBVex, BJABex, and LCL1ex) were isolated by differential centrifugation, as previously described (25). The protein concentrations of exosomes were determined using the Bio-Rad Dc assay, according to the manufacturer’s instructions. Three batches of exosome preparations (20 μg) were tested for endotoxin levels using the Limulus Amebocyte Lysate assay (Charles River Laboratories), and the following mean levels were detected: DG75-Coex (0.253 EU/ml), DG75-LMP1ex (0.076 EU/ml), and DG75-EBVex (0.273 EU/ml). Exosomes were phenotyped by flow cytometry after adsorption onto 4.5-μm precoated anti–MHC class II Dynabeads (clone HKB1, custom made; Dynal Biotech ASA/Invitrogen) overnight at room temperature at a concentration of 0.8 μg exosomes/9.5 × 10^5 Dynabeads for each staining in PBS containing 0.1% BSA and 0.01% sodium azide.

Exosomes coated on beads were stained with mouse monoclonal FITC-conjugated Abs (BD Pharmingen or BioLegend/Nordic Biosite) against human CD9 (M-L13), CD19 (4G7), CD21 (B-l4), CD23 (M-L233), CD40 (5C3), CD63 (MEM-259), CD80 (2D10), CD81 (JS-81), CD86 (2331), HLA-DR (L243), HLA-ABC (W6/32), IgG1 (MOPC-21), and IgG2a (MOPC-173). A total of 5 × 10^6 exosome-coated beads was acquired using a FACS-Calibur (Becton Dickinson), and data were analyzed using FlowJo software (TreeStar).

Nanoparticle tracking analysis

The size distributions of B cell–derived exosome preparations were analyzed by measuring the rate of Brownian motion using a NanoSight LM10 system, equipped with a fast video capture and particle-tracking software NTA 2.2. Exosome preparations were measured in triplicates at a concentration of 5 × 10^7 particles/ml.

Immunoblot analysis

DG75 cells (2 × 10^6) or exosomes (10 μg) were separated by SDS-PAGE (10%) and transferred to polyvinylidene difluoride membranes (Millipore). A total of 1 × 10^6 negatively selected B cells was incubated (37°C, 5% CO2) for 15 h with 100 μg BJABex or LCL1ex in 500 μl complete medium (48-well plate; Becton Dickinson). B cells were washed three times with PBS to remove unbound exosomes and incubated for the remaining 24 or 48 h in complete medium (37°C, 5% CO2). Cell lysates were separated by SDS-PAGE (NuPAGE 4–12% Bis-Tris Gel; Life Technologies) and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were stained according to the manufacturer’s instructions with Abs against LMP1 (CS. 1.4; Dako), EBNA2 (PE2; Novocastra), HLA-DR (L243), HLA-ABC (W6/32), IgG1 (MOPC-21), and IgG2a (MOPC-173). A PKH67 dye pellet centrifuged in parallel with labeled exosomes served as negative background control. Immunoblots were stained with the following Abs to distinguish B cells (CD3+ CD19+HLA-DR+), monocytes (CD3+CD14+HLA-DR+), and T cells (CD3+CD19+HIV); CD19-ECDF (HD237; B4 lytic; Beckman Coulter); HLA-DR-PE-Cy5 (TU36; BD Biosciences), CD14-PE (HCID4; BioLegend), and CD3 Pacific Blue (SP542; BD Biosciences). Exosome binding to live PBMCs (LIVE/DEAD Fixable Aqua Dead Cell Stain Kit; Invitrogen) was measured (∼130,000 events) using an LSR Fortessa (BD) or FACS Aria (BD) and analyzed using FlowJo software.

Binding pattern of exosomes to B cells and monocytes in PBMCs

PBMCs were isolated from buffy coat preparations of healthy blood donors (Blood Transfusion Center Solna, Stockholm, Sweden) through Ficoll–Paque Plus separation (GE Healthcare), as previously described (25). Exosomes (10 μg) were stained with a PKH67 Green Fluorescent Cell Linker Kit (Sigma-Aldrich), as previously described (25). Prefiltered (0.22-μm filter) PKH67-stained exosomes were added to PBMCs (2.5 × 10^5) for 1, 2, or 4 h at 37°C, 5% CO2. A PKH67 dye pellet centrifuged in parallel with labeled exosomes served as negative background control. PBMCs were stained with the following Abs to distinguish B cells (CD3 + CD19 + HLA-DR +), monocytes (CD3 + CD14 + HLA-DR +), and T cells (CD3 + CD19 + HIV); CD19-ECDF (HD237; B4 lytic; Beckman Coulter); HLA-DR-PE-Cy5 (TU36; BD Biosciences), CD14-PE (HCID4; BioLegend), and CD3 Pacific Blue (SP542; BD Biosciences). Exosome binding to live PBMCs (LIVE/DEAD Fixable Aqua Dead Cell Stain Kit; Invitrogen) was measured (∼130,000 events) using an LSR Fortessa (BD) or FACS Aria (BD) and analyzed using FlowJo software.

Human primary B cell isolation

B cells were isolated from PBMCs of healthy blood donors (Blood Transfusion Center Solna or Banc de Sang i Teixits, Barcelona, Spain). B cells were isolated either through negative selection (B Cell Isolation Kit II; Miltenyi Biotec) or by positive selection using biotinylated anti-IgD Ab (Southern Biotech) and Anti-Biotin MicroBeads (Miltenyi Biotec). The purity and B cell composition of each donor were assessed by flow cytometry, staining for CD19-allophycocyanin (HB19; BD), IgD-FITC (IA6-2; BD), CD38-PECy7 (HB7; BD), CD27-PE (M-T271; BD), and DAPI (5.7 μM; Sigma-Aldrich) using an LSR II or Fortessa (BD) and analyzed using FlowJo software (TreeStar).

EBV infection of primary human B cells

Negatively selected B cells were incubated with B95-8 virus–containing supernatant for 1.5 h, with shaking every 30 min (37°C, 5% CO2). Thereafter, the cells were washed with PBS (3 × 30 min, 300 × g) and resuspended in complete medium at a concentration of 2 × 10^6 cells/ml. Three days postinfection, supernatants were collected and centrifuged at 3000 × g for 30 min before storage at −80°C.
Confoical laser scanning microscopy analysis

A total of 3 \times 10^{5} negatively selected B cells (purity > 90%) was incubated in 250 \mu l complete medium with 40 \mu g PKH67-labeled exosomes in polypropylene tubes (Becton Dickinson) for 4 h at 37°C (5% CO_{2}). Cells were washed twice with PBS (400 \times g, 5 min) and fixed with 2% formaldehyde (Merck) for 10 min at room temperature. Cells were washed twice and incubated with purified human Ig (Sigma-Aldrich) and anti-CD19 (HB19; BD Pharmingen) for 30 min at room temperature. Washed cells were incubated with a secondary Ab Alexa Fluor 564 (Invitrogen) for 30 min at room temperature. Cells were washed and centrifuged (Cytospin3; Shandon) on microscopy slides (Menzel-Glaser), and VECTASHIELD HardSet Mounting Medium (Vector Laboratories) was used. For each sample, 150 cells were analyzed for surface-bound or internalized PKH67-labeled exosomes by confocal laser scanning microscopy (CLSM) (Leica TCS SP2 AOBS).

B cell apoptosis assay

Negatively selected B cells were used with a purity > 93% (n = 4; two donors in Barcelona and two donors in Stockholm). A total of 1.8 \times 10^{5} B cells was cultured in complete medium for 24 h in 200 \mu l (96-well round-bottom plates; BD). Cells were either left unstimulated or stimulated with soluble MegaCD40L (100 ng/ml; Enzo Life Sciences) and IL-21 (50 ng/ml; Enzo Life Sciences) or with 12.5 \times 10^{3} B cells (1.2 \times 10^{5} cells/200 \mu l) and IL-21 (100 ng/ml) or with 12.5 \mu g DG75 exosomes. RNA from 5 \times 10^{5} B cells was extracted (High Pure RNA Isolation Kit; Roche) and transcribed into cDNA (TaqMan Gold RT-PCR Kit; Applied Biosystems). Expression of AICDA (forward, 5’-AGAGGCTGA-CAGTGCTACA-3’; reverse, 5’-TGATGCCGAGGAAGACAAAT-3’) was investigated using a Bio-Rad CFX96 cycler. For each reaction, 250 nM primers, 10 ng cDNA, and 13 \mu l IQ SYBR Green Supermix (Bio-Rad) were used and run for 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. All reactions were standardized to the expression of EF-1\alpha (forward, 5’-CTGAACATCGACCGCAAAAT-3’; reverse, 5’-GGCGTTGTGCAAACTC-3’) and GAPDH (forward, 5’-GAAGGTGAGGTCCGAGGTACAC-3’; reverse, 5’-CAGAGTAAAAAGCAGCCTGTG-3’). Primers were purchased from TAG Copenhagen A/S.

Ig class-switch recombination analysis

RNA was extracted (High Pure RNA Isolation Kit; Roche) from 5 \times 10^{5} positively selected IgD⁺ B cells. The RNA was retrotranscribed (TaqMan Gold RT-PCR Kit; Applied Biosystems), and cDNA was used as a template to amplify isotype-specific I-Cμ circle transcripts (Iγ1/Cμ) and germline Iγ-Cμ transcripts (Iγ-Cμ and Iγ1/Cγ1) by PCR. Amplified PCR products were separated in a 1.5% agarose gel and transferred overnight onto nylon membranes (Amersham Biosciences) by Southern blot. Membranes were hybridized with appropriate radiolabeled probes, as reported (26, 27).

Statistical analysis

Statistical analysis was performed using Prism version 5.02 (GraphPad). The D’Agostino–Pearson omnibus test was used as a normality test. Normally distributed data were analyzed further using one-way ANOVA and the parametric unpaired Student t test, whereas nonnormally distributed data were analyzed using the nonparametric Mann–Whitney U test. The p values \leq 0.05 were considered significant.

Results

DG75-LMP1ex contain physiological levels of LMP1 as found on exosomes released during primary EBV infection

Exosomes from monoclonal EBV-transformed B cell lines (LCLs) contain high levels of LMP1 (19). However, whether these expression levels are physiological and are achieved during natural EBV infection remained to be elucidated. Therefore, we infected human peripheral B cells with EBV and isolated exosomes from cell culture supernatants 3 d postinfection. LMP1 levels in exosomes from uninfected or EBV-infected peripheral B cells (PBex and PB-EBVex) from two donors were compared with levels found in exosomes derived from the EBV⁺ Burkitt’s lymphoma cell line (BIALex) and LCL1 cells (LCL1ex). Immunoblot analysis revealed that PB-EVex from both donors harbored LMP1 (Fig. 1A). However, these levels were much lower than those in LCL1ex. Next, we screened exosomes from B cell lines in search of exosomes that would harbor lower amounts of LMP1, thereby better reflecting the physiological concentration observed in PB-EVex. We found that exosomes from the human DG75 Burkitt’s lymphoma cell line stably transfected with LMP1 (DG75-LMP1ex) harbored lower amounts of LMP1 compared with LCL1ex (Fig. 1B). No LMP1 expression was found in BIALex, the EBV⁺ DG75 Burkitt’s lymphoma cell line (DG75-COex), or its EBV-transformed subline (DG75-EBVex). LMP1 levels in exosomes reflected expression levels in the corresponding B cell line (Supplemental Fig. 1A). In line with their endosomal origin, all B cell–derived exosomes contained tetraspanin CD81 and HLA-DR molecules. Thus, we concluded that exosomes from DG75-LMP1 harbor similar LMP1 levels as those observed during primary EBV infection and that DG75 exosomes were suitable to elucidate their potential effect on human B cells.

DG75 exosomes harbor phenotypic differences that reflect the phenotype of their B cell line

Next, we further compared the phenotype of the DG75 cell lines (DG75-CO, DG75-LMP1, and DG75-EBV) and their corresponding exosomes (DG75-COex, DG75-LMP1ex, and DG75-
DG75 exosomes bind with similar efficiency to B cells in PBMCs and are internalized by B cells

To elucidate a functional effect of DG75-LMP1ex on human B cells, we first addressed whether different DG75 exosomes have similar binding capacities to human B cells. Therefore, exosomes were stained with the lipid dye PKH67, and their binding pattern to PBMCs was analyzed after 1, 2, and 4 h by multicolor flow cytometry (Fig. 3A). All DG75 exosomes showed increased binding to B cells and monocytes over time, and no statistical difference among DG75-COex, DG75-LMP1ex, and DG75-EBVex was detected (Fig. 3B). After 4 h, the binding efficiency for DG75 exosomes to B cells was 55–70% and to monocytes was 79–89%. Consistent with our previous study on exosomes derived from the LCL1 cell line, DCs, and human breast milk (25), all three DG75 exosomes showed a very low binding efficiency to T cells (<3%; data not shown).

Having found that DG75 exosomes bind with similar efficiency to human B cells, we next investigated whether exosomes are also internalized by the cells. Thus, we performed a kinetic study in which either no exosomes (–) or BJABex or LCL1ex harboring high levels of LMP1 were added to primary B cells for 24 or 48 h (Fig. 3C). To ensure maximal uptake but minimize the likelihood of detecting associated or unbound exosomes, B cells were washed extensively with PBS after 15 h. LMP1 was detected by immunoblot analysis in B cells incubated with LCL1ex at both time points. The two LMP1-specific bands have a molecular mass of 57–66 kDa and 50–55 kDa, corresponding to full-length and truncated LMP1 (19, 28). Yet to visualize internalization of exosomes, DG75 exosomes were labeled with the lipid dye PKH67 and incubated with primary B cells for 4 h at 37˚C. CLSM analysis revealed the intra- and extracellular localization of DG75 exosomes in B cells (Fig. 3D). A stronger and more frequent intracellular staining of PKH67+-exosome-positive B cells was observed for DG75-LMP1ex (~20%) compared with DG75-COex (~11%) and DG75-EBVex (~11%) (Fig. 3D). In summary, these findings indicated that DG75 exosomes bound with similar efficiency to B cells in PBMCs and were internalized by B cells.

DG75 exosomes do not prevent early apoptosis, but they induce B cell proliferation in PBMCs

Exosomes were demonstrated to shuttle proteins and RNAs to recipient cells in various settings, thereby influencing the cellular response (29). Having found that human B cells internalize DG75 exosomes, we wondered whether exosomes might provide survival signals. Therefore, B cells were incubated for 24 h with DG75-COex, DG75-LMP1ex, or DG75-EBVex and subsequently stained for Annexin V and propidium iodide (PI) to investigate signs of apoptosis (Fig. 4A). After 24 h, unstimulated (co) and IL-21 + CD40L–stimulated B cells already made up 53 and 41% of early apoptotic and late apoptotic/necrotic cells, respectively. No statistical difference in induction of apoptosis was observed when the addition of DG75 exosomes was compared with unstimulated B cells (early apoptotic, $p = 0.305$; late apoptotic/necrotic, $p = 0.781$; $n = 4$). Interestingly, we observed the formation of clumps in DG75 exosome–stimulated B cells in a similar manner as observed in CD40L– and IL-21 + CD40L–stimulated B cells (Fig. 4B). However, no difference was detected among the various DG75 exosomes. The observed clump formation prompted us to investigate in a first attempt whether DG75 exosomes have a functional impact and might induce the proliferation of lymphocytes. CFSE-labeled PBMCs were either left unstimulated (co) or stimulated with PHA or DG75 exosomes, and cell proliferation was assessed after 5 d by flow cytometry. The addition of DG75 exosomes to PBMCs did not induce proliferation of T cells, but it induced strong proliferation of B cells (Fig. 4C).
DG75 exosomes induce a dose-dependent proliferative response in B cells

The observed B cell–specific proliferation in PBMCs induced by DG75 exosomes prompted us to investigate whether DG75 exosomes also induce proliferation of isolated B cells. In particular, we wondered whether LMP1 transferred through DG75-LMP1ex might induce stronger proliferation in the recipient B cells than did DG75-COex and DG75-EBVex. Hence, B cells were labeled with CFSE, and proliferation was assessed by flow cytometry 5 d after stimulation with the different DG75 exosomes, alone or in combination with IL-21 (Fig. 5A). Synergistic activation of B cells with IL-21 + CD40L induced proliferation rates ranging from ~40–95%, depending on the blood donor. Because of this observed variability among the blood donors, all data were normalized to the proliferation rate of IL-21 + CD40L–stimulated...
B cells, which was set to 100% (Fig. 5B). CD40L stimulation alone induced lower proliferation rates (average, 33%) compared with the synergistic activation. In contrast, unstimulated (co) or IL-21–stimulated B cells did not proliferate (average, 2%). The addition of DG75 exosomes induced a dose-dependent proliferative response. Compared with unstimulated B cells, a significant increase in proliferation was observed when 25 μg of DG75-COex (12%) and DG75-LMP1ex (24%) were added, and a trend toward increased proliferation of DG75-LMP1ex compared with DG75-COex (p = 0.057) was noted. The addition of IL-21 to DG75 exosome stimulation did not increase the proliferation rates (Fig. 5B). Taken together, our data demonstrate that DG75 exosomes induce proliferation of human B cells in a concentration-dependent manner.

**FIGURE 3.** DG75 exosomes bind with similar efficiency to B cells in PBMCs and are internalized by B cells. (A) Gating strategy used to assess binding of PKH67-labeled exosomes, after 4 h, to human B cells and monocytes within PBMCs, showing representative contour plots. (B) Binding pattern of PKH67-labeled DG75-COex, DG75-LMP1ex, and DG75-EBVex, after 1, 2, or 4 h, to B cells and monocytes. Results from three (1 h) and five (2 or 4 h) independent experiments are shown. Horizontal lines represent the mean. (C) Human primary B cells were incubated for 24 or 48 h with no exosomes (○), BJABex, or LCL1ex, and cell lysates were assessed by immunoblot analysis for expression of LMP1 and β-actin. One representative experiment of three is shown. (D) Confocal microscopy analysis of anti-CD19(red) B cells incubated with PKH67-labeled (green) DG75 exosomes for 4 h at 37°C. Areas in the white boxes are shown in the Merge column. Arrows point to intracellular (green) localization of exosomes within B cells. Scale bars, 30 μm (left and middle panels), 7.5 μm (right panels). One representative experiment of three is shown.

DG75-LMP1ex induces differentiation into a CD19+CD38high CD20low plasmablast-like B cell population

Proliferating B cells have two fates in a germinal center reaction: differentiation into memory B cells or Ab-secreting plasmablasts (30). Hence, we addressed whether the observed proliferation is accompanied by B cell differentiation. CFSE-labeled B cells were stained for CD19, CD20, and CD38 expression. Plasmablast differentiation is characterized by increased expression of CD38 and decreased expression of CD20 (Fig. 6A). Synergistic activation with IL-21 + CD40L for 5 d gave rise to a CD19+CD38highCD20low population with an average of 11% compared with an average of 6% observed in unstimulated B cells (Fig. 6B). Addition of 5 μg...
DG75 exosomes did not induce an increase in that population; however, the addition of 25 μg of DG75-LMP1ex induced a significant increase, with an average of 26% of the CD19⁺CD38highCD20low population compared with unstimulated B cells (Fig. 6B). In contrast, addition of 25 μg of DG75-COex and DG75-EBVex induced, on average, only 12% of the CD19⁺CD38highCD20low population. As already observed in the proliferation assay (Fig. 6B), the addition of IL-21 did not enhance the differentiation effects induced by the exosomes alone. These data suggest that DG75-LMP1ex induce differentiation into a CD19⁺CD38highCD20low plasmablast-like cell population.

**DG75 exosomes induce class-switch recombination in human IgD⁺ B cells**

A key feature of activated B cells is that they undergo class-switch recombination (CSR) that diversifies the effector function of the secreted Ab. A hallmark of active CSR is upregulation of the enzyme AID, the formation of looped-out circular DNAs (circle transcripts), and germline transcription (31). Intrinsic LMP1 expression was shown to induce CSR from constant μ (Cμ) to multiple Cγ, Ca, and Ce genes in a NF-κB–dependent manner (27). For this reason, we investigated whether B cells stimulated with DG75 exosomes showed signs of active CSR. First, we measured the upregulation of AID (AICDA) transcripts by quantitative real-time PCR in IgD⁺-selected B cells exposed to DG75 exosomes, alone or in combination with IL-21 (Fig. 7A). B cells stimulated with DG75 exosomes plus IL-21 upregulated AICDA transcripts comparable to IL-21 + CD40L–stimulated B cells. Next, we addressed whether DG75 exosomes induced the formation of circular IgY1/2-Cμ transcripts, as well as IgY1/2-Cγ1 germline transcription. Of note, the formation of looped-out cir-
circular DNA detected by circle transcript formation precedes germline transcription (31). Amplified Ig1/2-Cmc circular transcripts were detected by Southern blot analysis in primary B cells when stimulated with IL-21, CD40L, IL-21 + CD40L and when exposed to DG75-COex, DG75-LMP1ex alone, and DG75-EBVex + IL-21 (Fig. 7B). Ig1/2-Cy1 germline transcription was observed in CD40L, DG75-LMP1ex, DG75-EBVex, and IL-21 + DG75-COex or DG75-LMP1ex B cells of another donor (Fig. 7C). Altogether, these data indicate that DG75 exosome stimulation induces circle transcript formation and germline transcription in primary human B cells.

Discussion

In this study, we provide evidence that B cell–derived exosomes shuttle functional information to human B cells and, thereby, influence B cell biology. The DG75 Burkitt’s lymphoma cell line (DG75-CO), LMP1-transfected cell line (DG75-LMP1), or EBV-infected cell line (DG75-EBV) were used as constitutive sources for B cell–derived exosomes, with the hypothesis that they might mimic exosomes released during EBV infection or EBV-associated diseases. Our data demonstrate that DG75 exosomes efficiently bind to B cells within PBMCs, are internalized by B cells, and induce proliferation and CSR. Additionally, we observed that DG75-LMP1 exosomes are able to induce the differentiation of B cells into a plasmablast-like phenotype.

LCLs are characterized by high expression of LMP1 that facilitates the outgrowth and survival of a particular clone within the EBV-infected polyclonal B cell culture. Constitutive LMP1 signaling is limited through association of endogenous LMP1 with endosomal tetraspanin CD63 and subsequent secretion via exosomes (18). Our results showed that primary EBV-infected B cells also released exosomes harboring LMP1, but expression levels were much lower compared with LCL-derived exosomes (Fig. 1A). Instead, the Burkitt’s lymphoma cell line stably transfected with LMP1 (DG75-LMP1) was a suitable source to obtain human exosomes that harbored LMP1 at physiological concentrations and, thus, potentially mimic exosomes that are released during primary EBV infection (Fig. 1B).

Primary human B cells stimulated with IL-4 plus anti-CD40 secrete exosomes that reflect the activation state of the B cells (32). Consistent with these findings, DG75 exosomes reflected the phenotype of their corresponding B cell line (Fig. 2B). Ectopic LMP1 expression in EBV Burkitt’s lymphoma cell lines was shown to increase MHC class I and II Ag expression (33, 34). In line with this, DG75-LMP1ex had significantly higher levels of HLA-ABC and HLA-DR than did DG75-COex (Fig. 2B). In general, it has to be stressed that all three DG75 exosomes had a phenotypic profile that distinguished them, and these differences are likely to influence biological effects. For instance, DG75-LMP1ex and DG75-EBVex had significantly higher levels of HLA-ABC molecules compared with DG75-COex, and it is tempting to speculate that they contain EBV-specific peptides that could be presented on the surface of DCs or B cells after their uptake. Potentially, these exosomes could be an “additional source” of viral peptides, which increase the frequency of EBV-specific CTLs. In contrast, increased expression of HLA-DR molecules on DG75-LMP1ex compared with DG75-COex and DG75-EBVex might be an additional Ag source used by DCs to license CD4+ Th cells that, in turn, can activate B cells, thereby inducing Ab responses. Also, LMP1 was detected only in DG75-LMP1ex; the diverse effects seen in this study between the different DG75 exosomes are clearly not only dependent on the presence of LMP1 (Fig. 1B). Of note, the low or undetectable
LMP1 levels in DG75-EBV cells and DG75-EBVex, respectively, are in agreement with a previous study (24).

A large body of evidence indicates that exosomes play a major role in intercellular communication and, thereby, influence the outcome of an immune response (1, 35). To contribute to intercellular communication, exosomes have to interact with and deliver their content to the recipient cell. In a previous study, we observed that DC- and breast milk–derived exosomes had a different binding pattern within PBMC cultures compared with exosomes from a gp350-expressing LCL (LCL1) (25). Our data demonstrate that the different DG75 exosomes bound with similar efficiency to B cells and monocytes within PBMC cultures (Fig. 3B). Furthermore, the detection of LMP1 shuttled through LCL1ex in B cell lysates indicated exosome binding and suggested their uptake (Fig. 3C). Confocal microscopy analysis demonstrated internalization of DG75 exosomes by B cells (Fig. 3D). Recently, fusion of the exosomal membrane with the plasma membrane was demonstrated as a mechanism by which functional miRNA shuttled by DC-derived exosomes is delivered to the acceptor DC (36). Pegtel et al. (29) demonstrated the functional delivery of mature EBV-encoded microRNAs via exosomes released from EBV-infected B cells to monocyte-derived DCs. However, it still has to be elucidated which uptake mechanism, direct fusion or endocytosis, allows B cell–derived exosomes to deliver their content into the cytoplasm of the recipient B cell.

Apoptosis plays a critical role in B cell development and homeostasis, and the T cell–derived cytokine IL-21 was shown in vitro to induce apoptosis of resting and activated primary murine B cells (37). Consistent with that, unstimulated and IL-21 + anti-CD40–stimulated primary human B cells also showed signs of apoptosis and necrosis already at 24 h, and the addition of DG75 exosomes did not protect resting B cells from apoptosis (Fig. 4A). Ectopic LMP1 expression in a B cell line and EBV infection of IgD+ B cells in vitro provide B cell survival signals through upregulation of autocrine BAFF and a proliferation-inducing ligand (APRIL) expression (27, 38). However, the concentration of LMP1 in B cells after delivery through DG75-LMP1ex is much lower compared with ectopically expressed LMP1 used in the above-described study (27). Future studies will investigate whether exosomes carrying high amounts of LMP1 induce autocrine BAFF and APRIL expression that provide survival and proliferation signals to B cells. This might be of particular interest in providing a link between EBV exosomes and SLE, in which increased BAFF expression rescues self-reactive B cells from peripheral deletion (39).

The addition of DG75 exosomes to PBMC cultures induced proliferation in B cells, whereas no proliferation was seen for T cells (Fig. 4C). It has to be stressed that the absence of T cell proliferation might be due to the very low binding efficiency of DG75 exosomes to T cells (3%; data not shown). A dose-dependent proliferation was observed when isolated B cells were exposed to DG75 exosomes, with a trend toward increased proliferation for DG75-LMP1ex (Fig. 5B). We would like to point out that these data were generated in two laboratories with consistent results (Sweden and Spain). Compared with isolated B cells, B cell proliferation within PBMCs was much stronger, indicating the presence of APCs, CD4+ T cell help, and solvable factors released by these cells is important to boost B cell proliferation (Figs. 4C, 5B). The proliferative capacity is supported by the observation that DG75 exosomes are taken up by B cells, as well as the more pronounced intracellular staining of DG75-LMP1ex by CLSM (Fig. 3D). Moreover, it suggests that DG75-LMP1ex delivered functional LMP1 that can signal through TNFR-associated factor adaptor molecules to govern proliferation in recipient B cells. Our data are in line with the finding that EBV-mediated B cell proliferation is dependent upon LMP1, as well as the observation of increased development of lymphoma in LMP1-
transgenic mice (40, 41). However, it remains to be elucidated which proliferation-inducing factor is delivered by DG75-COex and DG75-EBVex. The expression of EBNA2 and LMP1 is essential for EBV transformation of B cells in vitro (42, 43). Immunoblot analysis of cell lysates from DG75 cells revealed no expression of EBNA2 (Supplemental Fig. 1A). This is in accordance with previous reports, namely that the original cell line DG75-CO is EBV− and that EBV infection did not induce EBNA2 expression (22, 24). Therefore, we can rule out that EBNA2 is delivered via DG75 exosomes to B cells.

In contrast, the question arises which B cell population proliferated after exposure to high doses of DG75 exosomes. Negatively isolated peripheral B cells were used as recipient cells, which consist of naïve (IgD−CD27−), marginal zone (IgD−CD27+), and memory (IgD−CD27+) B cells (44, 45). Preliminary data on isolated IgD+ B cells also revealed a dose-dependent proliferation of DG75 exosomes, with increased proliferation for DG75-LMP1ex (C. Gutzeit, unpublished observations). Thus, it is likely that the responding cell population is either naïve and/or circulating marginal zone B cells. Strikingly, the proliferating B cells exposed to DG75-LMP1ex differentiated into a CD19+CD38hiCD20low plasmablast-like population (Fig. 6). Human IgD−CD27+ marginal zone B cells were shown to have increased capacity to differentiate and to secrete all IgG subclasses compared with naïve B cells (46). Therefore, future studies will focus on the ability of exosomes to stimulate this particular B cell subset.

To mount protective immune responses, B cells diversify Ig-encoding genes through CSR, which is mandatory for the maturation of the Ab response and crucially requires AID (47). Stimulation of IgD+ B cells with DG75 exosomes + IL-21 induced the upregulation of AID transcripts (Fig. 6A). Recently, it was demonstrated that BCR signaling needs to synergize with TLR signaling to induce AID and T cell–independent CSR (48, 49). Our data suggest that DG75 exosomes might provide a yet unknown primary CSR-inducing signal (e.g., BCR crosslinking), which then synergizes with cytokine signaling to induce AID. Additionally, hallmarks of active CSR are the formation of circular transcripts and germline transcription (31). Germline transcripts play a central role in CSR by directing AID to a specific S region within the IgH locus, and IL-21 was shown to be a switch factor for Cγ1 and Cγ3 transcripts in human B cells (50, 51). Stimulation of IgD+ B cells with DG75 exosomes induced the formation of Ig1/2-Cμ circle transcripts, as well as Ig1/2-Cγ1 germline transcript (Fig. 7A, 7B). Ectopic LMP1 expression in a BJAB cell line stably transfected with a tetracycline-inducible LMP1 expression vector was shown to induce Ig1/2-Cγ1 germline transcripts (27). However, it remains to be investigated further why the synergistic stimulation of IgD+ B cells with DG75 exosomes plus IL-21 did not increase circle transcript formation and germline transcription.

In conclusion, our study demonstrates the B cell–stimulatory capacity of exosomes released by EBV-infected B cells. So far, various studies have only elucidated an immune-suppressive effect of these exosomes on recipient cells, such as human T cells and DCs (15, 29). However, B cells are equipped with all mandatory adaptor molecules to provide signaling for viral proteins, such as LMP1, a mimic of the B cell–activating receptor CD40 (16). Thus, we propose that B cell–derived exosomes released from EBV-infected B cells are able to deliver their content to B cells and, thereby, influence B cell biology. Therefore, clinical features observed in patients with EBV-associated diseases, such as lymphoproliferative disorders or autoimmune diseases, might be intensified by the presence and action of these exosomes. Furthermore, they might influence B cell development in healthy EBV carriers with implications, for example, for allergy or autoimmune disease development.

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Disclosures

The authors have no financial conflicts of interest.

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