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Induction of Exosome Release in Primary B Cells Stimulated via CD40 and the IL-4 Receptor

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Exosomes are lipid-bound nanovesicles formed by inward budding of the endosomal membrane and released following fusion of the endosomal limiting membrane with the plasma membrane. We show here that primary leukocytes do not release exosomes unless subjected to potent activation signals, such as cytokine or mitogen stimulation. In particular, high levels of exosomes were released when murine splenic B cells were stimulated via CD40 and the IL-4 receptor. This property was shared by B cells from different anatomical locations, as newly formed, marginal zone and follicular B cells were capable of secreting exosomes upon CD40/IL-4 triggering. B cell exosomes expressed high levels of MHC class I, MHC class II, and CD45RA (B220), as well as components of the BCR complex, namely, surface Ig, CD19, and the tetraspanins CD9 and CD81. Ig on the plasma membrane of primary B cells was targeted to the exosome pathway, demonstrating a link between the BCR and the exocytic pathway. IgD and IgM were the predominant Ig isotypes associated with CD40/IL-4 elicited exosomes, though other isotypes (IgA, IgG1, IgG2a/2b, and IgG3) were also detected. Together, these results suggest that exosome release is not R constitutive activity of B cells, but may be induced following cell: cell signaling. The Journal of Immunology, 2008, 180: 8146–8152.

In this study, we sought to determine whether exosomes, or other forms of sMHC were released from primary leukocytes. We analyzed the release of sMHC from leukocytes subjected to a variety of mitogenic signals, including TLR ligands, cytokines, and CD40 triggering. The highest levels of sMHC triggered in primary splenocytes were released from CD40- and IL-4-stimulated B cells and the sMHC was exclusively associated with exosomes. B cell-derived exosomes were enriched in both MHC-I and MHC-II and expressed Ig, as well as several other components of the BCR complex. IgD and IgM present at the plasma membrane of primary B cells was shuttled into the exosome pathway, demonstrating linkage of the surface BCR to the exosome pathway in B cells that receive T cell help. Together, these results demonstrate that exosome release is not constitutive activity of B cells, but is only induced following the provision of certain cellular activation signals.

Materials and Methods

Mice and human peripheral blood

BALB/c mice obtained from The Jackson Laboratory were maintained and bred in specific pathogen-free conditions at the Otago University Heretics/ Taieri Unit. Animal studies were approved by the regional Animal Ethics Committees. Peripheral blood was obtained by venesection with the approval of the University of Otago Human Ethics Committee. Mononuclear cells were isolated by Ficoll-Hypaque (Sigma-Aldrich) centrifugation as previously described (6).

Cells, mitogens, and cytokines

Splenocytes were isolated from BALB/c mice and cultured in RPMI 1640 plus 10% FCS (R10) supplemented with streptomycin, penicillin (Invitrogen), and 50 μM 2-ME in 6- or 24-well plates (Nunc) at 2 × 10^6/ml. Splenocytes were stimulated with the following mitogens: PHA (5 μg/ml), Con A (2.5 μg/ml), PMA (25 ng/ml), ionomycin (750 μg/ml), Leuconostoc spp. dextran sulfate (M, 500 kDa; 15 μg/ml), Excherichia coli LPS (1 μg/ml), Staphylococcus aureus lipoteichoic acid (5 μg/ml), and staurosporine (250 nM). All reagents were sourced from Sigma-Aldrich. Surface Ig was cross-linked using divalent goat anti-mouse Ig (1 μg/ml, Southern Biotechnology Associates). IL-4 (BD Pharmingen) was used at 50 ng/ml. CD40 (5 μg/ml, clone FKG-45 (7), was provided by A. Rolink, Center for Biomedicine, Basel, Switzerland). CD3 (1 μg/ml, 145-2C11; American Type Culture Collection), CD28 (1 μg/ml, clone 37-51, provided by J.

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Allison, Memorial Sloan Kettering Cancer Center, New York, NY). Neutralizing mAb to CD 40 ligand (CD40L, clone MR1 (8); BD Biosciences), and anti-IL-4 (clone 11B11 (9), provided by J. Kirman, Malaghan Institute, Wellington, NZ) were included in splenocyte cultures at 10 μg/ml. mAbs were purified from hybridomas grown in a CellLine1000 Bioreactor (Integra Biosciences) by ammonium sulfate precipitation or protein G affinity purification. Bone marrow-derived dendritic cells (BMDC) were generated exactly as previously described (5). Human B cells were transformed by EBV using the conditioned culture supernatant of the marmoset B95/8 cell line. Due to a cross-reaction of the sHLA-DR ELISA with soluble marmoset MHC-II present in B95/8 culture supernatants, we first incubated 1 ml of 10^7 PBMC with 1 ml of B95/8 supernatant for 4 h in 24-well plates in the presence of 1 μg/ml cyclosporin A (Novartis). Cells were then resuspended and washed twice in warm (R10), resuspended in medium plus cyclosporin A, and returned to the original culture wells.

Ultracentrifugation and exosome isolation

Splenocytes, BMDC, or the A20 cell line were cultured for 3 days in RPMI 1640 plus particulate-depleted 10% FCS. We were unable to confirm the presence of exosomes in FCS (data not shown). However, because numerous protein aggregates were visualized in 100,000 × g FCS pellets by transmission electron microscopy, FCS was subjected to a 16 h, 100,000 × g step to clear debris. Culture supernatants were first spun at 400 ωg for 20 min to deplete cells and then at 10,000 × g for 20 min to deplete residual cellular debris. Samples were then 10,000 × g ultracentrifuged for 1 h at 4°C. In some experiments, 0.1% sodium deoxycholate (DOC) was added at room temperature 1 h before gel filtration or ultracentrifugation to release lipid-bound MHC Ags from exosomes. Continuous sucrose gradient separation of exosomes was carried out as previously described (1, 10).

Abs, CFSE staining, flow cytometric analysis, and sorting

The following Abs were used: TCRβ-PE (PE; clone H57-597), anti-I-A^b (AMS-32.1), anti-I-Eβ (14-4-4), anti-I-A/E (2G9), CD5-PE (HI1086), CD9-bio (KMC8), CD11c-bio (HL3), CD11b-bio (M1/70), CD19-bio (1D3), CD21-bio (7G6), CD23-bio (B3B4), CD27-bio (LG.3A10), CD29-bio (Ha2/5), CD40-PE (3/23), CD44-PE (IM7), CD45-PE (M1/42; American Type Culture Collection; biotin-conjugated in house). Accurate Type Culture Collection; biotin-conjugated in house). Allophycocyanin-conjugated to streptavidin (BD Biosciences) was used to detect biotinylated Abs. To detect membrane-bound Ig, highly cross-absorbed donkey anti-murine Ig conjugated to PE (Jackson ImmunoResearch Laboratories; Australian Laboratory Services) was resuspended at 10 μg/ml in PBS plus 0.5 μg/ml rat Ig and applied to cell or exosome suspensions. For flow cytometric analysis of exosomes, 10 μg of exosomes (isolated by 100,000 × g ultracentrifugation) in 100 μl of PBS was added to 25 μl of aldehyde sulfate microspheres (1.4 × 10^9/ml) and incubated overnight at 4°C. Microspheres were blocked by the addition of 0.5 ml of 100 mM glycine/PBS (pH 7.4) and incubated at room temperature 1 h before gel filtration or ultracentrifugation to release lipid-bound MHC Ags from exosomes. Continuous sucrose gradient separation of exosomes was carried out as previously described (1, 10).

Immunoprecipitation and Western blotting

To investigate the entry of surface Ig into the exosomal pathway, RBC lysed splenocytes (2.5 × 10^6/ml) were surface biotinylated with 1 mg/ml NHS-LC-Biotin (catalog no. 21335; Pierce and Global Science) for 10 min at 4°C. Cells were then washed twice in 100 mM glycine/PBS (pH 7.4) and resuspended in 0.5 ml of 0.5 M NaCl, 100 mM Tris-HCl (pH 8.0) with Complete protease inhibitor mixture; Roche) and Ig was immunoprecipitated from lysed exosomes (100 μg of protein in 100 μl lysis buffer) using 15 μl of 10× concentrated BioMag goat anti-mouse Ig beads (Polysciences). Beads were rotated overnight at 4°C and washed twice in lysis buffer, once in 0.25% Nonidet P-40/PBS, and once in PBS. Reduced and boiled precipitates (note, samples used for CD9, CD63, and CD81 analysis were not reduced or boiled) were then separated by 12% Bis-Tris-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 1% BSA. Biotinylated H and L chains of Ig were detected by streptavidin-HRP (1:1000 in 0.1% casein/PBS). For immunoblotting, we blocked membranes with 0.1% casein and used rabbit antisera recognizing the cytoplasmic domains of I-Aα and I-Aβ at lysed in lysis buffer (0.5% Triton X-100, 0.25% CHAPS, 150 mM NaCl, 100 mM Tris-HCl (pH 8.0) with Complete protease inhibitor mixture; Roche) and Ig was immunoprecipitated from lysed exosomes (100 μg of protein in 100 μl lysis buffer) using 15 μl of 10× concentrated BioMag goat anti-mouse Ig beads (Polysciences). Beads were rotated overnight at 4°C and washed twice in lysis buffer, once in 0.25% Nonidet P-40/PBS, and once in PBS. Reduced and boiled precipitates (note, samples used for CD9, CD63, and CD81 analysis were not reduced or boiled) were then separated by 12% Bis-Tris-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 1% BSA. Biotinylated H and L chains of Ig were detected by streptavidin-HRP (1:1000 in 0.1% casein/PBS). For immunoblotting, we blocked membranes with 0.1% casein and used rabbit antisera recognizing the cytoplasmic domains of I-Aα and I-Aβ at

![FIGURE 1. Induction of sMHC-II (s-Aβ) release in splenocytes stimulated with various agents. A, Splenocytes (2 × 10^6 in 1 ml of medium) were incubated in 24-well plates with the indicated reagents. After 3 days, culture supernatants were analyzed for the levels of s-Aβ by ELISA (B) and the number of viable B cells by trypan blue counting along with flow cytometric analysis of CD19^+ B cells (C). B, Splenocytes were stimulated with CD40 and IL-4 and the number of viable B cells and soluble I-Aβ release was determined by ELISA at the indicated times. C, Splenocytes were labeled with CFSE and cultured with the indicated reagents. After 3 days, proliferation of CD19^+ B cells was determined by flow cytometry. Dot plots show total splenocytes and histogram shows CD19^+ gated B cells. Thin line, Unstimulated cells; thick line, stimulated cells. D, sMHC-II secretion in CD3/CD28-stimulated cultures is dependent on CD40L and IL-4 signaling: splenocytes were stimulated with CD3 and CD28 mAb in the presence of the 5 μg/ml of the indicated neutralizing mAb for 3 days and soluble I-Aβ levels were determined by ELISA. E, Human PBMC were stimulated with the indicated reagents for 5 days before determining the levels of soluble HLA-DR in the culture supernatants. F, Human PBMC were infected with EBV and soluble HLA-DR was determined by ELISA at the indicated times. Controls were PBMC cultured in medium alone (Nil) or in medium plus cyclosporin A (Cyc A). Representative of four experiments.](http://www.jimmunol.org/doi/abs/10.4049.jimmunol.1701022)
An I-Ad-specific ELISA. Representative of four experiments. Supernatants were removed from cultures and analyzed for sMHC-II using 280 nm chrome 240 kDa; human albumin, 65 kDa; deoxyribonuclease, 37 kDa; cyto-

standard compounds of known mass (Blue Dextran, 2000 kDa; catalase, sMHC was estimated by comparison of the elution volumes with those of 100,000 U/ml is equivalent to the signal of detergent (DOC). Exosomal pellet fractions were resuspended in PBS (equal to the original centrifuged volume). Pre- and postcentrifugation supernatant and pellet samples were then analyzed by ELISA for soluble I-A^d-. B, Culture supernatants were harvested at day 3 from splenocytes cultured with CD40 and IL-4 or CD3 and CD28 as indicated. Supernatants were fractionated by 100,000 x g ultracentrifugation in the absence or presence of detergent (DOC). Exosomol pellet fractions were resuspended in PBS (equal to the original centrifuged volume). Pre- and postcentrifugation supernatant and pellet samples were then analyzed by ELISA for soluble I-A^d-. B, Culture supernatants were harvested at day 3 from splenocytes cultured with CD40/IL-4 (left) or CD3/CD28 (right). Supernatants were fractionated by FPLC on a Sepharose column in the absence or presence of detergent (DOC). Elution of sMHC-II in the eluate was determined by I-A^d-specific ELISA. C, Pellets prepared from 100,000 x g ultracentrifugation of the supernatants of 3-day CD40/IL-4-stimulated B cell cultures were fractionated by sucrose density gradient and PAGE-separated proteins were then immunoblotted for the I-A^d-chain. D, Transmission electron microscopic visualization of day 3 negatively stained exosomes from CD40/IL-4-stimulated B cells cultures isolated by 100,000 x g ultracentrifugation. Representative of four experiments.

FIGURE 2. Identity of cells releasing soluble MHC-II in response to CD40/IL-4 triggering and requirement for both T and B cells in CD3/CD28-mediated soluble MHC-II release: A and B, splenocytes were sorted by flow cytometry into CD19^+ B cells and TCR^B+ T cells and stimulated either alone or in subset combinations in the presence of the indicated reagents for 3 days and levels of soluble I-A^d were determined by ELISA. C. Newly formed, follicular (Fo) and marginal zone (MZ) B cell subsets release sMHC-II in response to CD40/IL-4 triggering. B cells were gated for CD19 and sorted using the indicated gates into CD19^+CD21^low CD23^low newly formed B cells (NF), CD19^+CD21^lowCD23^low marginal zone B cells, CD19^+CD21^lowCD23^high follicular B cells, or total CD19^+ B cells (labeled CD19^+ in D) and stimulated with CD40/IL-4. D. At day 3, supernatants were removed from cultures and analyzed for sMHC-II using an I-A^d-specific ELISA. Representative of four experiments.

1/500 dilution, a gift from Dr. J. Villadangos, Walter and Eliza Hall Institute, Melbourne, Australia). The rabbit anti-calnexin, CD63 (LAMP3), and Tsg101 were all from Santa Cruz Biotechnology (Global Science).

1/500 dilution, a gift from Dr. J. Villadangos, Walter and Eliza Hall Institute, Melbourne, Australia). The rabbit anti-calnexin, CD63 (LAMP3), and Tsg101 were all from Santa Cruz Biotechnology (Global Science). HRP-conjugated goat anti-mouse Ig whole molecule (A-4416) was from Sigma-Aldrich.

Soluble MHC and Ig ELISA

Soluble murine or human MHC-II was detected by double determinant ELISA, exactly as previously described (5). OD readings were quantified into unit values against a standard curve prepared from LPS-treated BMDC and secreted Ig was determined using the mouse monoclonal isotyping reagents (ISO-2; Sigma-Aldrich).

Gel permeation

Gel permeation fast protein liquid chromatography (FPLC) was conducted using a Superose 12 HR 10/30 column (GE Healthcare Bio-Sciences) at a constant flow rate of 0.5 ml/min using PBS as the eluant. Culture supernatants were clarified by differential centrifugation, filtered (0.22 μm), and 0.5 ml injected onto the column. Fractions (0.7 ml) were collected and analyzed by soluble I-A^d ELISA (see above). The molecular mass of sMHC was estimated by comparison of the elution volumes with those of standard compounds of known mass (Blue Dextran, 2000 kDa; catalase, 240 kDa; human albumin, 65 kDa; deoxyribonuclease, 37 kDa; cytochrome c, 15 kDa; vitamin B12, 1.35 kDa) monitored by absorbance at 280 nm.

Transmission electron microscopy and immunolabeling

Exosomes isolated by 100,000 x g ultracentrifugation were applied to formvar-coated copper grids. The formvar surface was rendered hydrophilic before use by exposure to plasma discharge in an Edwards E306A vacuum coating system (Edwards High Vacuum). The exosomes were negatively stained with 1% phosphotungstic acid (pH 7.0) and the grids were visualized using a Philips CM100 BioTWIN transmission electron microscope (Philips/FEI) and images were captured using a MegaView III digital camera (Olympus Soft Imaging Solutions).

Results

Exosome release by primary cells

To determine the possible hematopoietic source of the circulating MHC identified in earlier studies (5, 13–15), we cultured splenocytes in medium alone or in the presence of stimulatory cytokines, mitogens, TLR ligands, or anti-Ig and measured sMHC release over several days by ELISA. Although several mitogens were able to induce the release of modest amounts of sMHC-II, the highest levels of sMHC were released by B cells stimulated with agonistic

FIGURE 3. Characterization of released sMHC-II as exosomal. A and B, Culture supernatants were harvested at day 3 from splenocytes cultured with CD40 and IL-4 or CD3 and CD28 as indicated. Supernatants were fractionated by 100,000 x g ultracentrifugation in the absence or presence of detergent (DOC). Exosomal pellet fractions were resuspended in PBS (equal to the original centrifuged volume). Pre- and postcentrifugation supernatant and pellet samples were then analyzed by ELISA for soluble I-A^d-. B, Culture supernatants were harvested at day 3 from splenocytes cultured with CD40/IL-4 (left) or CD3/CD28 (right). Supernatants were fractionated by FPLC on a Sepharose column in the absence or presence of detergent (DOC). Elution of sMHC-II in the eluate was determined by I-A^d-specific ELISA. C, Pellets prepared from 100,000 x g ultracentrifugation of the supernatants of 3-day CD40/IL-4-stimulated B cell cultures were fractionated by sucrose density gradient and PAGE-separated proteins were then immunoblotted for the I-A^d-chain. D, Transmission electron microscopic visualization of day 3 negatively stained exosomes from CD40/IL-4-stimulated B cells cultures isolated by 100,000 x g ultracentrifugation. Representative of four experiments.
Phenotype of B cells and B cell-derived exosomes analyzed by flow cytometric data can be found in Table I.

Identification of APCs responsible for sMHC release
To more precisely determine the source of released sMHC-II, we sorted splenocytes into CD19⁺ B cells and TCRαβ⁺ T cells (Fig. 2A). Although CD19⁺ B cells alone were capable of secreting sMHC-II in response to CD40/IL-4, admixing of CD19⁺ B cells and CD40 mAb plus IL-4 (Fig. 1A). Soluble MHC-II release displayed similar kinetics to the proliferation of B cells (Fig. 1B) and, in general, soluble MHC release correlated with the number of viable cells present in the mitogen-stimulated splenocyte suspensions (Fig. 1A). An exception was dextran plus LPS which induced extensive B cell proliferation as determined by CFSE analysis of cell division (Fig. 1C). However, compared with CD40/IL-4 stimulation, dextran plus LPS induced 53.9% ± 26.5 SD (n = 5) of B cell proliferation, but only 7.0% ± 7.3 SD (n = 4) of soluble MHC-II release. The release of exosomes was also triggered in splenocytes by CD3 and CD28 stimulation (Fig. 1D). A combination of neutralizing Ab against CD40L and IL-4 reproducibly inhibited of sMHC-II release, demonstrating that CD3/CD28 stimulation of exosome release is at least partly dependent on CD40L and IL-4 signaling (Fig. 1D).

We next confirmed the results for humans using PBMC. PBMC showed a similar release of sMHC-II in response to several mitogens, CD40 plus IL-4, or following infection of B cells with EBV (Fig. 1, E and F). A variety of TLR ligands, namely, LPS, lipoteichoic acid, N-palmitoyl-S-[2,3-bis(palmitoyloxyl)propyl]cysteine, poly(I:C), peptidoglycan, flagellin, and CpG, failed to induce sMHC-II release in human or murine B cells (data not shown).

As a control for nonspecific release of sMHC by dying cells, splenocytes were rendered apoptotic by inclusion of staurosporine (Fig. 1, A and D). Despite the induction of extensive death in splenocytes, sMHC-II was not released from apoptotic cells.

Table I. Expression of surface markers on fresh or stimulated B cells and anti-CD40 mAb/IL-4 or anti-CD3/anti-CD28 mAb-elicited exosomes

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<th>CD40/IL-4</th>
<th>CD3/CD28 Exosomes</th>
<th>BMDC Exosomes</th>
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*Fresh or stimulated B cells and exosomes were analyzed for marker expression via flow cytometry, MHC-II⁺ refers to exosomes bound indirectly to aldehyde sulfate beads via anti-MHC-II mAb to ensure that only B cell-derived exosomes were analyzed. Exosomes secreted from the A20 B cell lymphoma line and BMDC are also shown for comparison. Shifts in the mean fluorescence intensity compared to isotype controls are represented as follows: mean fluorescent intensity (MFI) < 5 = −, MFI 5–10 = −/+ , MFI 10–50 = +, MFI 50–200 = +++, and MFI >200 = +++. All results are representative of two experiments.
with TCRαβ+ T cells was required for release of sMHC-II during CD3/CD28 stimulation (Fig. 2B). To determine whether there existed a heterogeneity in the sMHC-II release by different B cell subsets present in spleen in response to CD40/IL-4 stimulation, we isolated newly formed, follicular and marginal zone B cells by FACS, as previously described (11, 12) (Fig. 2C). All B cell subsets were capable of releasing sMHC-II in response to CD40/IL-4 triggering. However, in all four experiments, follicular B cells released 30–70% more sMHC-II than the other subsets (Fig. 2D).

sMHC released by splenocytes is bound to exosomes

Having established that B cells release sMHC in response to T cell-derived signals, we next sought to establish whether the sMHC was bound to membranes or vesicles, or was present as an oligomeric form, not associated with lipid-rich structures (5). Almost all sMHC pelleted at 100,000 × g and the observed sMHC fractionation into the pellet fraction was detergent sensitive (Fig. 3A). Gel filtration showed that almost all sMHC was present in the void volume of FPLC-fractionated, conditioned cell culture medium (Fig. 3B). DOC caused a shift in the molecular mass of sMHC-II from the void volume into fractions that corresponded to the expected molecular mass of dimeric MHC-II (~100 kDa). To further confirm that the released sMHC was exosome associated, we subjected 100,000 × g isolated exosomes to sucrose gradient ultracentrifugation and detected the MHC-II β-chain in the fractions by Western blotting. As expected, MHC-II-positive vesicles migrated to densities between 1.13 and 1.15 (Fig. 3C), consistent with densities previously reported for exosomes released by immortalized B cell lines (1, 16). We also examined 100,000 × g pellets by transmission electron microscopy and confirmed the presence of typical features of exosomes such as cup-shaped morphology and 50- to 120-nm diameter (Fig. 3D).

Phenotype of exosomes released by B cells

Exosomes released from primary B cells were analyzed by flow cytometry following coupling to aldehyde sulfate beads or by Western blotting. CD40/IL-4-elicited exosomes contained several surface markers in common with both freshly isolated (fresh) and CD40/IL-4-cultured B cells, including B220 (CD45R) and surface markers of the BCR complex (Fig. 4A and Table I). The absence of the endoplasmic reticulum molecule calnexin in exosome preparations demonstrated a lack of contamination of exosome preparations by cell debris containing endoplasmic reticulum membranes (Fig. 4B). Both the CD9 and CD81 tetraspanin markers associated with the BCR were found to be enriched on B cell-derived exosomes (Fig. 4); however, the tetraspanin CD63 (LAMP-3) was weakly detectable in B cell lysates, but absent from exosomes. Tsg101 has been reported to be expressed by exosomes released from the DC-like cell line D1 (17), but was only weakly expressed by either CD40/IL-4-stimulated B cells or exosomes. The CD21 BCR-associated protein was absent or only very weakly expressed, on exosomes, but was expressed by both fresh and CD40/IL-4-cultured B cells (Fig. 4A). High levels of MHC-I and MHC-II were present on the surface of B cell-derived exosomes (Fig. 4). Analysis of costimulator molecule expression showed that CD80 was absent or only weakly expressed by B cell exosomes, but moderate levels of CD40 and CD86 were detected (Table I). CD3/CD28-elicited exosomes contained a similar composition of surface markers as compared with CD40/IL-4 exosomes; however, TCRαβ was found to be expressed by CD3/CD28 exosomes, suggesting contamination with T cell-derived exosomes. However, when only MHC-II+ exosomes were analyzed from CD3/CD28-stimulated splenocytes using anti-MHC-II mAb-coated beads to bind exosomes, CD3/CD28 elicited exosomes were found to be devoid of TCRαβ and CD5, markers expressed by the majority of splenic T cells (Table I). These results suggest that both T cell- and B cell-derived exosomes are secreted from CD3/CD28-stimulated splenocytes. Interestingly, Ig was detected on exosomes by flow cytometry and its expression was confirmed by Western blotting (Fig. 4B and Fig. 5B) and isotype-specific ELISA (Fig. 5A). Although CD40/IL-4-stimulated B cells secreted a range of soluble, Ig isotypes (IgA, IgG1, IgG2a/2b, and IgG3) as nonmembrane-bound forms into the supernatant, IgM and IgD were found to be the predominant isotypes enriched on the surface of exosomes (Fig. 5).

Plasma membrane Ig is targeted to exosomes

To determine whether surface Ig was capable of entering the exosome pathway, we surface biotinylated B cells before stimulation with CD40/IL-4. After 3 days, exosomes were isolated and Western blotting was used to confirm that biotinylated, plasma membrane-derived IgM and IgD H chains, as well as Ig L chains, were present in the exosome preparations (Fig. 5B). In contrast, the IgG H chain band was present at only low intensity. We next tested whether the trafficking of Ig into the exosome pathway was specific to the IgM and IgD isotypes or was determined by the density of surface expression of Ig isotypes on B cells. To distinguish between these two possibilities, we biotinylated the cell surface of
the IgG2a-positive A20 B cell lymphoma line and precipitated Ig from released exosomes. As shown in Fig. 5B, A20 exosomes contained biotinylated IgG2a derived from pools at the cell surface. This suggests that the entry of Ig into the exosome pathway is primarily determined by the density of Ig present on the cell surface, rather than isotype-specific factors.

Discussion

Earlier work has clearly shown that immortalized B cells are prodigious producers of exosomes (1); however, our study shows that cellular activation is critical for exosome release from primary cells. Surprisingly, LPS plus dextran induced substantial proliferation in B cells (Fig. 1, A and C) but failed to induce exosome release on a par with CD40/IL-4. Exosome synthesis was similarly induced in human B cells by EBV infection, although this effect was less pronounced than with CD40/IL-4 stimulation. This may relate to slower kinetics of exosome biosynthesis by viral infection or simply reflect the low frequency of B cells able to be infected by EBV in our in vitro cultures. These results suggest that immortalization of primary B cells by viral infection also provides signals for the induction of exosome release.

The combination of CD40 and IL-4 has previously been shown to induce B cell proliferation (13–15). Continuous stimulation of B cells with CD40L and IL-4 leads to long-term proliferation of B cells, but CD40L/IL-4 B cell lines are not immortal and remain factor dependent (18). IL-4- and CD40-stimulated human B cells are potent APCs, with similar potential for immunotherapy as DC (19, 20). Our results now show that CD40/IL-4-treated primary B cells possess a similar exosome secretory mechanism to DC (21, 22).

Our results using neutralizing Abs clearly demonstrate that IL-4 and CD40L signaling are required for release of exosomes from B cells incubated with CD3/CD28-stimulated T cells. NKT cells express CD40L and produce large amounts of IL-4 when triggered via the TCR and thus were also candidates for stimulating exosome release in B cells. However, similar levels of exosomes were released from B cells within cd1d−/− splenocyte suspensions devoid of NKT cells (Ref. 23 and data not shown). This is consistent with our sorting experiments (Fig. 2A) demonstrating that conventional TCRαβ+ T cells provide the main source of CD40L and IL-4 required for triggering exosome release in B cells. Interestingly, IL-4 triggers exosome release in mast cells, suggesting that IL-4 may be a common trigger for exosome release in distinct cell types (24).

Surface Ig (mostly IgD and IgM) on primary B cells and IgG2a present on a class-switched B cell line was targeted to exosomes. These findings are consistent with the recent data of Rialland et al. (25) showing that IgG was targeted to exosomes in a human lymphoma cell line. However, in their system, cross-linking of the BCR was required, a stimulus previously shown to induce endocytosis of surface Ig and redistribution into MHC-II-rich endosomal vesicles (26, 27). Similarly, naïve B cells triggered with anti-Ig plus CD40 mAb were recently reported to traffic surface-derived MHC-II into the exosome pathway, although the transfer of Ig into the exosome pathway was not investigated in this report (16).

Our findings that CD40 and IL-4 signaling switches on a program of exosome synthesis in primary B cells suggest that exosome synthesis is a process tightly regulated by T cell-derived stimuli. Thus, exosomes would only be released once B cells have received help from CD4+ T cells. Due to a low precursor frequency of Ag-specific B cells early in the immune response, it is likely that only low numbers of exosomes could be released at early time points. However, T cell help induces extensive B cell proliferation: at day 3 in our experiments, CD40 and IL-4 had induced up to five cell divisions within the first 3 days, representing a 32-fold expansion in some subsets (Fig. 1C). Thus, amplification of exosome release by Ag-specific B cells might occur early in the humoral immune response. Likely targets of B cell-derived exosomes identified in our study are follicular DC. Exosomes were reported to decorate the surface of follicular DC in human tonsils in vivo and B cell line-derived exosomes were shown to bind to isolated follicular DC (28). Due to the inflamed nature of the tissue obtained from routine tonsillectomies, it seems likely that follicular DC acquired the exosomes from in vivo-activated B cells present in the tissue used as the source of follicular DC.

We have previously demonstrated that circulating MHC-I and -II in normal individuals is present as a simple dimeric form not associated with exosomes (5). Our present results do not clarify the source of circulating sMHC in serum of normal animals, but suggest that exosomal Ag may be released only during an immune response. Given the rapid removal of vesicles by the liver (29), we predict that released exosomes would display a very short half-life in the circulation, but may be of importance during cellular communication between closely apposed cells, such as T cells, B cells, and follicular DC or conventional DC in lymphatic organs. Importantly, our results show that predictions of exosome release from resting, primary cell types cannot always be determined from studies of their malignant or immortal counterparts, such as B cell lines. Furthermore, this work is in agreement with a recent report showing that resting B cells require activation for exosome release (16). Together, these findings emphasize that cell signaling is an essential mechanism of exosome synthesis in primary cells.

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Disclosures

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