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Exosomes from Bone Marrow Dendritic Cells Pulsed with Diphtheria Toxoid Preferentially Induce Type 1 Antigen-Specific IgG Responses in Naive Recipients in the Absence of Free Antigen

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Exosomes derived from dendritic cells (DC) activate T cells in vivo, but whether exosomes are able to induce and/or modulate humoral immune responses is still unknown. We show that murine bone marrow DC pulsed in vitro with an intact protein (diphtheria toxoid (DT)) produce exosomes that induce, in the absence of free protein, in vivo Ig responses specific for DT in naive recipients. Furthermore, these exosomes stimulate secondary IgG anti-DT responses in mice primed with intact DT. Exosomes from mature, relative to immature, DC were more effective at inducing primary, although not secondary, IgG anti-DT responses. Whereas intact DT preferentially induced a type 2 (IgG1) anti-DT response, exosomes from DT-pulsed bone marrow DC favored induction of type 1 (IgG2b and IgG2a) DT-specific IgG. These results are the first to demonstrate the ability of exosomes derived from Ag-pulsed DC to induce and modulate Ag-specific humoral immunity in vivo. The Journal of Immunology, 2006, 177: 3757–3762.

Although the ability of the DC-derived exosomes to prime T cells has been studied in detail (5–7), their ability to induce humoral immune responses remains unclear. The importance of this point is underscored by the potential use of exosomes as vaccines, including those targeting bacterial infections in which humoral immunity plays a dominant role (12, 13). Thus, exosomes derived from an intestinal epithelial cell line cultured with predigested OVA and treated with IFN-γ primed mice for secondary IgG and IgE anti-OVA responses following s.c. immunization with OVA in CFA (14). However, the ability of exosomes to induce a primary anti-OVA Ig response was not tested. A single report demonstrated the ability of exosomes from an immortalized DC line, pulsed with a Toxoplasma gondii Ag extract, to prime mice for specific Ig responses after experimental infection with the parasite (13). However, the role of exosomes in inducing Ig responses was unclear, because repetitive injections with exosomes and challenge with the parasite were used.

In this report, we study the ability of exosomes from diphtheria toxoid (DT)-pulsed mature and immature bone marrow DC (BMDC) to induce DT-specific Ig responses during an inflammatory response. We demonstrate, for the first time, that exosomes, containing DT processed to serologically undetectable levels, induce a primary IgM and IgG anti-DT response in vivo, and that the maturation of the DC producing the exosomes increases their ability to induce primary IgG anti-DT responses. These anti-DT responses were biased toward type 1 IgG isotypes (IgG2b and IgG2a), in contrast to the preferential type 2 isotype, IgG1 induced by intact DT. Thus, exosomes derived from DC can induce and modulate humoral immunity through a type 1 polarizing mechanism.

Materials and Methods

Mice

BALB/c mice were obtained from The Jackson Laboratory and used at 8–10 wk of age for the experiments. Mice were maintained in a pathogen-free environment at Uniformed Services University of the Health Sciences.

The experiments in this study were conducted according to the principles set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.
Production and culture of BMDC

DC were cultured from bone marrow of BALB/c mice in the presence of 10 ng/ml GM-CSF as described previously (15), using culture medium supplemented with 6% FCS that was ultracentrifuged two times at 100,000 × g for 18 h at 10°C for depletion of bovine exosomes and protein aggregates.

Preparation of exosomes

BMDC were cultured at 1 × 10^7 cells/ml in the presence or in the absence of 30 μg/ml detoxified DT provided by Dr. J. Mond (Biosynexis, Gaithersburg, MD). After 24 h of culture, both DT-pulsed and unpulsed BMDC were stimulated with 20 ng/ml LPS to induce DC maturation or maintained in the absence of LPS, followed by addition of another 30 μg/ml DT to the DT-stimulated BMDC cultures. After another 24 h of culture, BMDC culture supernatants were collected and cell debris was removed by centrifugation at 300 × g for 10 min at 4°C. Cleared culture supernatants were filtered through 0.22-μm cellulose acetate filters (GE Osmonics). Exosomes were 60–90 nm in diameter and filter freely through 0.22-μm filters. We did not observe significant losses of tetraspan-containing microvesicles after filtration. Filtered supernatants were centrifuged at 10,000 × g for 30 min, and the exosomes collected from the supernatant at 100,000 × g for 1 h at 4°C, washed with cold PBS, and then resuspended in PBS at a 1:500 volume. Sterility was preserved throughout the process. Purified exosomes contained less than one viable bacteria per 25 μg of protein. Protein content was determined by the bicinchoninic acid method (Pierce).

Exosomes purified from culture supernatants of BMDC pulsed with DT are referred to as “DT-exosomes,” and those from BMDC cultured in the absence of DT as “control-exosomes.” Preparations obtained from LPS-stimulated BMDC cultures are referred to as “mature control- or DT-exosomes,” and those from cultures lacking LPS as “immature control- or DT-exosomes.” A similar preparation obtained from culture medium is referred to as “Media” and was used to control for the potential contaminant carryover during the purification. This preparation contained no detectable protein (<0.6 μg/ml).

Coupling of exosomes to latex beads and immunofluorescence analysis

A total of 10^7 aldehyde/sulfate polystyrene latex beads (Interfacial Dynamics), 3.9 μm in diameter, was coated with 1 μg of exosomes for 16 h at 20°C, washed, and incubated for 1 h with 1% BSA. For FACS analysis, 50,000 latex beads coated with exosomes were incubated with 0.2 μg of fluorochrome-conjugated mAbs specific for several DC markers or isotype-matched negative control mAbs. Samples were analyzed in triplicate. All mAbs used were obtained from BD Pharmingen, except for anti-CD9 mAb (clone Nyn.H3), which was obtained from Emfret. Latex beads incubated with the Media preparations and blocked with BSA were used as a control for nonspecific binding of mAb to beads. Only single beads were gated for analysis. Acquired data were analyzed using the WinList3D program. The mean fluorescence intensity of the phenotypic markers was used to compare their relative expression in mature and immature exosomes.

Quantitation of bovine contaminants in the preparations of exosomes

Bovine haptoglobin (Hp) was quantified essentially as previously reported for human Hp (16) using both a direct adsorption ELISA and a capture ELISA with affinity-purified rabbit anti-bovine Hp (Immunology Consultants Laboratory). Hp purified from fresh bovine acute-phase serum (Life Technologies) was used as a reference, typically giving an OD_{405} of 3.0. Guinea pig sera and wells not incubated with anti-CD63 mAb were used as negative controls. More than 93% of the detectable CD9-CD63 vesicles were pelleted by a single ultracentrifugation of the FCS.

Bovine microvesicles were detected by a sandwich-capture ELISA using two mAbs specific for bovine tetraspans CD9 and CD63 (detect). Therefore, the ELISA detects CD9-CD63 complexes, but not free CD9 or CD63. Wells were coated with 1 μg/ml purified mouse IgG2 anti-bovine CD9 mAb (Abcam; clone IV500, Abcam), blocked with 2% BSA in PBS, and incubated overnight at 4°C with exosomes. Captured CD9-containing vesicles were detected with a mouse IgG1 anti-bovine CD63 mAb (clone CC25; Serotec). Bovine FCS (not ultracentrifuged) used as a reference, typically gives an OD_{520} of 3.0. Guinea pig sera and wells not incubated with anti-CD63 mAb were used as negative controls. Approxi- mately 93% of the detectable CD9-CD63 vesicles was pelleted by a single ultra-centrifugation of the FCS at 100,000 × g for 18 h.

Quantitation of marine cytokines

IL-6 and IL-12 concentrations in the purified exosomes were measured using a quantitative sandwich ELISA previously described in detail (15).

Quantitation of DT

The content of intact DT in the purified exosomes was determined by competitive inhibition ELISA. Briefly, wells coated with 5 μg/ml DT, and blocked with 2% BSA, were incubated with serial 2-fold dilutions of puri-fied DT standard or DT-exosomes diluted in a 1/200,000 dilution of a high-titrated mouse anti-DT sera. Control-exosomes were used as negative controls. The Ig binding was detected with a goat anti-mouse IgG conjugated to AP. The limit of detection of the assay was 5 ng/ml DT. DT was also quantified in exosomes following treatment for 1 h at room temperature with 1/5 Triton X-100 (TX-100). Both samples and standards were treated with detergent during incubations.

Mouse immunizations

Groups of 20 female BALB/c mice at 8–10 wk of age were immunized i.p. with 250 μl of an emulsion of CFA and PBS or CFA and a solution of 25 μg of DT in PBS at day 0. Twenty-four days later, five mice of each group received one i.v. injection of 25 μg of mature or immature DT-exosomes or 25 μg of mature or immature control-exosomes in 200 μl of PBS. Additional groups of mice immunized with CFA or DT plus CFA emulsion received at this time (day 24) one i.v. injection with an equal volume of media, or with 25 pg of LPS and 0.02 μg of intact DT in PBS, two times the upper limit of DT that could be detected in a 25-μg dose of exosomes. All mice were bled at day 0 to obtain preimmune sera, and at days 14, 23, 38, and 44 for measurement of anti-DT Ig isotype titers by ELISA.

Measurement of serum titers of Ig anti-DT isotypes

DT-coated and 2% BSA-blocked ELISA plates were incubated with serial 3-fold serum dilutions overnight at 4°C. Replicate plates were then incubated for 1 h at 37°C with polyclonal goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM, or IgG (γ-chain) conjugated to alkaline phosphatase (Southern Biotechnology Associates). Titors were expressed as the dilution of sera giving an absorbance at 405 nm equal to 1.0, following the development of the enzymatic reaction for 1 h, using p-nitrophenyl-pyrophosphate as substrate. Preimmune sera were used as negative controls.

Statistics

Data were expressed as arithmetic mean ± SEM of the individual titer. Levels of significance of the differences between the groups were determined by the Student’s t test. Values of p < 0.05 were considered statistically significant.

Results

Purified exosomes from BMDC express high levels of Ag-presenting and costimulatory molecules

Our major goal was to determine the ability of BMDC-derived exosomes to induce Ag-specific humoral immunity in vivo, following preincubation of BMDC cultures in vitro with a soluble protein. DT, a highly immunogenic protein widely used as a carrier in conjugate vaccines, was selected as our model protein (17, 18). BMDC were cultured in the presence or in the absence of DT for 48 h, and the exosomes were then purified from the culture supernatant. This early time point in the culture was selected to reduce the risk of contamination with apoptotic microvesicles, because BMDC undergo some spontaneous apoptosis following the first
48 h of culture (19). Exosomes from these immature BMDC showed high levels of tetraspans (CD9 and CD81), predominant in exosomes, CD11b and one of its ligands (CD54), but very low levels of CD11c (Fig. 1). These exosomes also expressed relatively high levels of MHC-II, MHC-I, CD1d, and CD86, with CD80 expressed at lower levels (Fig. 1). Because exosomes from immature BMDC have been reported to have anti-inflammatory (9) and even T cell-immunosuppressive properties (8), we wanted to compare these immature exosomes with those obtained from BMDC stimulated during the last 24 h of culture with 20 ng/ml LPS. This stimulation induced BMDC maturation as evidenced by up-regulation of cell surface MHC-II and CD86 (data not shown). Exosomes secreted from LPS-matured BMDC, showed an identical expression level of tetraspans relative to immature BMDC, consistent with the attachment to the beads of equal numbers of exosomes, but demonstrated an increased expression of MHC-II, CD86, CD80, and especially of CD54 (Fig. 1). CD54 has been shown to be critical for priming of naive T cells (4). The increased expression of these makers was similar in two independent experiments run in triplicate (p < 0.01). The phenotype of the control-exosomes obtained from BMDC cultured in the absence of DT, was identical with that shown for DT-exosomes in Fig. 1, suggesting the pulse with DT do not affect the phenotype of the secreted exosomes. Collectively, these data suggest that exosomes will have immunostimulatory properties that may be enhanced by BMDC maturation.

**Purified exosomes are essentially free of bovine contaminants**

Next, we determined the level of medium-derived protein contaminants within the purified exosomes, because these contaminants could produce artifacts following mouse immunization. Three bovine proteins: tetraspan (CD9/CD63) complexes, Hp, and BSA, were used as tracers. Tetraspans are highly expressed in cell membranes and were thus used as markers of contamination for bovine vesicles. Bovine tetraspans were readily detectable in de-complemented FCS, but following the first and second ultracentrifugation of FCS used for BMDC culture, >93% and essentially 100% of the detectable tetraspans were removed, respectively. Furthermore, bovine tetraspans were undetectable in BMDC-derived exosomes (Table I), suggesting that all the purified exosomes were murine.

HP present at low concentrations in FCS (1.6–2.6 ng/ml), is readily polymerized by heating, generating very high m.w. aggregates that could be copurified with exosomes (12). Thus, HP is a useful protein marker to follow both purity and copurification of aggregated proteins. Aggregated HP was completely removed following FCS ultracentrifugation (<0.3 ng/ml) and was undetectable in the purified exosomes (<0.005% of total protein; Table I), suggesting that aggregated bovine proteins are not a significant contaminant of our preparations of purified exosomes.

Ultracentrifugation removed <14% of total BSA, which is a major component of FCS (>600 μg/ml culture medium). In this regard, BSA was detected in the purified exosomes used in the experiments illustrated in Figs. 1–3 (Table I), although it was <0.065% of the total protein. A similar content of BSA was detected (0.42 ± 0.05 ng/μg of protein; n = 32) in other preparations of exosomes. The tendency of BSA to copurify with exosomes has been observed (12). Collectively, these data suggest that, although some major protein component of FCS may be copurified in very low but quantifiable amounts, we can consider our BMDC-derived exosomes as being essentially free of bovine contaminants.

**Purified exosomes derived from BMDC pulsed with DT do not contain serologically detectable DT**

Next, we wanted to determine the potential presence of serologically intact DT within the purified exosomes. Using a high-titered mouse anti-DT sera in a competitive inhibition ELISA, we determined that DT was not serologically detectable (<0.04% protein or <10 ng of DT in 25 μg of exosomes) in either intact (Table I) or 1% TX-100 solubilized (data not shown) control- or DT-exosomes. The pulse of BMDC with DT did not affect the yield or purity of the exosomes. LPS-matured BMDC consistently yielded lower amounts of exosomes than immature BMDC (Table I). Collectively, the purified preparations of immature exosomes (n = 6) yielded for control exosomes 0.82 ± 0.09 μg/ml supernatant, similar (p = 0.07) to that of DT-exosomes (0.76 ± 0.08 μg/ml supernatant). In contrast, the yield of mature exosomes was lower than for immature exosomes in both control exosomes (0.65 ± 0.07 μg/ml supernatant; p = 0.01) and DT-exosomes (0.59 ± 0.06 μg/ml supernatant; p = 0.008).

**Exosomes from DT-pulsed BMDC induce IgG DT-specific responses in naive recipients**

Immunization of naive mice with intact CFA plus DT induced DT-specific IgM and IgG responses with the delayed kinetics and isotype distribution typical of responses to T cell-dependent Ags (Fig. 2B). Thus, IgG anti-DT was the prevalent isotype, peaking at 24 days postimmunization. In contrast, IgM anti-DT titers were relatively low, peaked at day 14, and gradually decreased to undetectable levels by day 30. However, a major part of the decreased detection of IgM over the time is likely due to competition with IgG anti-DT, present in sera in overwhelming amounts, at time points later than 14 days. Sera from naive mice contained no detectable IgG anti-DT (titer <1/10) and CFA alone did not induce IgG anti-DT responses, or significantly affect preimmune natural IgM anti-DT levels (Fig. 2A).

We next determined the ability of exosomes, derived from either immature or mature DT-pulsed BMDC (DT-exosomes), to initiate primary Ig isotype responses in naive mice, and/or to boost secondary Ig responses in mice primed 24 days earlier with intact DT. Exosomes (25 μg of total protein) were injected i.v., without adjuvant, allowing exosomes direct access to the spleen in the absence of local inflammation. Exosomes purified from both DT-pulsed immature and mature BMDC induced primary IgM and IgG anti-DT responses, more sustained than that observed in naive

![FIGURE 1. Phenotype of exosomes. Latex beads coated with exosomes purified from the culture supernatant of BALB/c (MHC-II+) BMDC pulsed with DT in the absence (immature) or presence of 20 ng/ml LPS after 24 h of culture (LPS-matured) were analyzed by FACS. Latex beads coated with Media were used as controls. The histograms obtained for one of the negative isotype controls (rat IgG2b) is shown. The phenotype of the control-exosomes obtained from BMDC cultured in the absence of DT was identical with that shown for DT-exosomes.](image-url)
mice immunized with intact DT (Fig. 2A), and boosted the IgG anti-DT response in DT-immune mice (Fig. 2B). However, mature DT-exosomes induced a primary IgM anti-DT response (Fig. 2A) that was 10-fold higher than the IgG response induced by immature DT-exosomes (p < 0.0003), whereas the primary IgM (p > 0.11) and boosted IgG (p > 0.05) anti-DT responses were not significantly different between the two groups. In contrast, injection of Media or control-exosomes from immature (data not shown) or mature (Fig. 2) BMDC had no effect on these responses. These responses were unlikely due to free intact DT, because serologically detectable DT in the dose injected was <0.01 μg. Furthermore, we measured <1 μg of LPS per microgram of exosome protein and the injection of 25 μg of LPS and 0.02 μg of intact DT had no effect on these responses (Fig. 2). These results suggest that exosomes are a competent stimulus to initiate primary humoral Ag-specific responses, as well as to stimulate a secondary response in primed mice. Furthermore, the maturation state of the DC releasing the exosomes has a major impact in the efficiency of those exosomes to induce primary Ab responses in naive recipients.

IgG2b and IgG2a are the major subclasses induced by DT-exosomes, in contrast to the predominant IgG1 anti-DT response induced by intact DT

We next wanted to determine the IgG subclass distribution induced by either intact DT or DT-exosomes. Immunization with intact DT induced IgG responses that were mostly IgG1 (type 2 response). Thus, serum titers of IgG1 anti-DT were 30–100 times higher than those of IgG2a or IgG2b anti-DT at any given time point postimmunization (Fig. 3A). IgG3 was only a minor part of the IgG anti-DT response. Serum titers and kinetics for the IgG1 anti-DT response following immunization with intact DT, was very similar to that obtained for total IgG (Fig. 2).

In striking contrast, both immature and mature DT-exosomes induced primary DT-specific IgG responses that were predominantly IgG2b and IgG2a (type 1 response), with only a relatively modest contribution by IgG1 (3- to 5-fold lower serum titer than IgG2b or IgG2a) (Fig. 3A). As mentioned earlier for IgG, higher overall IgG isotype responses, with the exception of IgG3 (p = 0.056), were observed in response to mature, relative to immature, DT-exosomes. Thus, exosomes induce primary IgG responses in nonimmune recipients through a type 1-associated mechanism. This result lends strong support to the notion that exosomes are directly involved in the induction of IgG anti-DT responses, and not copurified traces of free DT.

In mice primed with intact DT, secondary immunization with mature DT-exosomes significantly boosted IgG1 anti-DT (p = 0.03–0.01), IgG2a anti-DT (p = 0.0001–0.000006), and IgG2b anti-DT (p = 0.006–0.003) responses relative to either mature or immature control-exosomes, Media, or 20 ng of intact DT plus LPS. In contrast, IgG3 titers were unaffected (p = 0.74–0.56). Immature DT-exosomes also boosted each anti-DT IgG subclass in a similar manner as the mature DT-exosomes (Fig. 3B; p = 0.87–0.19). Of interest, the IgG1/IgG2a anti-DT titer ratio, which was 76 ± 12, 21 days after primary immunization with intact DT, was essentially maintained 20 days after a subsequent injection with control exosomes (IgG1/IgG2a ratio, 51 ± 14; p = 0.14). However, following secondary immunization with either immature or mature DT-exosomes, this ratio was significantly reduced (IgG1/IgG2a ratio, 10 ± 2; p = 0.0002), suggesting that exosomes could mediate a relative shift to a type 1 response in mice previously primed for a type 2 response.

Exosomes do not contain detectable IL-12 or IL-6

BMDC activation with pathogen-associated molecular patterns results in secretion of proinflammatory cytokines, particularly IL-12 and IL-6 (15, 19). We observed >30 ng/ml of both IL-12 and IL-6 in BMDC culture supernatants after 24 h of stimulation with 20

Table I. Contaminants of the preparations of exosomes (nanograms/microgram of protein)

<table>
<thead>
<tr>
<th></th>
<th>Yield (µg/mg supernatant)</th>
<th>Bovine Hp</th>
<th>Bovine CD9/CD63</th>
<th>DT</th>
<th>Murine IL-12</th>
<th>Murine IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control exosomes</td>
<td>0.91±0.74±</td>
<td>&lt;0.05</td>
<td>OD_{405} &lt; 0.01</td>
<td>&lt;0.4</td>
<td>&lt;0.002</td>
<td>&lt;0.0004</td>
</tr>
<tr>
<td>DT-exosomes</td>
<td>0.83±0.62±</td>
<td>&lt;0.05</td>
<td>OD_{405} &lt; 0.01</td>
<td>&lt;0.4</td>
<td>&lt;0.002</td>
<td>&lt;0.0004</td>
</tr>
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a,b Range obtained in the preparations of exosomes from “immature” and “mature” BMDC used in the experiments depicted in Figs. 1–3.

a OD_{405} < 0.01 in 10 µg of exosomes by capture ELISA (undiluted FCS OD_{405} = 3.0).

FIGURE 2. DT-specific Ig isotype responses induced by exosomes derived from DT-pulsed BMDC. BALB/c mice (five mice per group) were immunized i.p. with an emulsion of CFA (A) or PBS (CFA (A) or B) containing 25 µg of DT (CFA + DT), and 24 days later, mice received an i.v. injection (indicated by arrows) of 25 µg of exosomes purified from the culture supernatant of BMDC loaded with DT and stimulated with LPS (mature DT-exosomes) or not (immature DT-exosomes), or from BMDC not exposed to DT but stimulated with LPS (mature control-exosomes). Additional control groups were injected with 25 µg of LPS plus 20 ng of intact DT. Data are expressed as the geometric mean ± SEM. Statistical significance between the Ig titers induced by DT-exosomes and control-exosomes is indicated by an asterisk (*) and between those induced by mature and immature DT-exosomes by a pound sign (#). The data shown are representative of two independent experiments.
ng/ml LPS (data not shown). IL-12 is a key type 1-polarizing cytokine produced by DC (20), whereas IL-6 can act directly on B cells to induce maturation to Ab secretion (21). IL-12 and IL-6 have been implicated in DC priming for antibacterial IgG responses (15). However, we were unable to detect either IL-12 or IL-6 in the intact (Table I) or 1% TX-100 solubilized exosomes (data not shown), arguing against their direct role in the exosome-mediated induction of type 1 polarizing activity and Ig induction, respectively.

Discussion

DC-derived exosomes express high levels of MHC and costimulatory molecules, which are up-regulated upon activation with pathogen-associated molecular patterns in the same way that occurs on the surface of the intact DC membrane. The release of exosomes may thus serve to amplify DC function through their immunostimulatory effects on more distant cell types. In this regard, exosomes derived from DC have been shown to induce in vivo priming of Ag-specific CD4+ and CD8+ T cells (5–7). Here, we show that exosomes also can induce primary Ag-specific Ig responses in naive mice to proteins that have been processed by the DC in vitro. These exosomes induce an immune deviation from an otherwise type 2 (IgG1-predominant) toward a type 1 (IgG2-predominant) response.

The mechanism by which exosomes mediate T cell activation is partially understood. Thus, it has been demonstrated that exosome-mediated T cell priming requires the participation of mature DC in the recipient host (5–7). Additionally, only exosomes from mature DC will trigger effector T cell responses in vivo (4). DC maturation is a key component of the innate, inflammatory response to a wide range of pathogens. Therefore, we hypothesized that, if exosomes in fact contributed to the initiation of primary Ag-specific Ig responses, activation of both the recipient APCs and the DC releasing the exosomes would be required. Thus, we initially induced a proinflammatory response systemically through i.p. immunization with a DT emulsion of CFA in PBS (naive mice). Secondary IgG subclass responses to mature control- or DT-exosomes are shown, because titers did not differ from those obtained in mice injected with the corresponding immature exosomes.

Discussion

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Mice receiving a single injection of DT-exosomes produced DT-specific primary IgM and IgG responses, showing kinetics of induction similar to that observed following immunization with intact protein. The maturation state of the DC producing the exosomes had a major impact in the magnitude of the primary anti-DT IgG responses, but not in the IgM responses. This enhanced ability of mature DT-exosomes to induce an IgG isotype response in resting Ag-specific B cells is likely due to its greater immunostimulatory properties via increased expression of costimulatory and Ag-presenting molecules (Fig. 1). However, we cannot rule out the possibility of improved targeting of the mature exosomes to competent recipient cells through increased expression of CD54 (Fig. 1) or other unidentified molecules. The greater efficiency of mature exosomes to induce primary IgG responses perfectly fits the model in which, during inflammation, the initiation and magnitude of the induced primary Ag-specific Ig response must be commensurate to the level of pathogen stimulation and consequently to the level of DC activation. Once the adaptive response is established, the maturation state of DC being stimulated will have less relevance. Thus, we do not observe significant differences in the ability of mature and immature exosomes to boost secondary IgG responses induced by intact DT.
Our findings suggest that, because the stimulation of T cells alone cannot induce Ag-specific humoral responses, not only T cells, but Ag-specific B cells were activated by DC-derived exosomes. Of interest, exosomes appear to mediate Ag-specific B cell activation with, at best, a minimal amount of intact protein Ag. Thus, using mouse DT-specific polyclonal antisera produced in response to immunization with intact DT, we were unable to detect DT in exosomes derived from DT-pulsed BMDC, even after detergent solubilization. Our preparations of DT-exosomes do not contain detectable levels of free or exosome-associated DT (<10 ng of intact DT per immunizing dose). Presuming that some intact DT-epitopes are required for the B cell-specific Ig response, these data suggest an extremely efficient mechanism for exosome-mediated immunity. B cell clones producing natural anti-DT IgM, could have served to amplify the DT responses. The injection of 20 ng of intact free DT (plus LPS) was unable by itself to initiate a primary IgG or IgM anti-DT response, suggesting that if these DT-exosomes are present, they must be physically linked with the exosomes in an immunogenic manner.

DT-exosomes, mature and immature, induced a primary anti-DT response in naive mice biased toward type 1 isotypes (IgG2b and IgG2a) in contrast to intact DT in which a largely type 2 (IgG1) response was observed. Similarly, DT-exosomes boosted anti-DT responses in mice primed with intact DT, coupled with an increase in the ratio of DT-specific IgG2a/IgG1. IL-12 was undetectable in our preparations of exosomes, leaving unresolved the mechanism by which exosomes induced type 1 polarization. Studies using IL-12−/− mice indicate that type 1 immunity can be induced in the absence of endogenous IL-12 (20), suggesting that exosome-mediated type 1 polarization might similarly occur through an IL-12-independent mechanism.

In summary, our data suggest that exosomes released by DC following exposure to and uptake of pathogens expressing TLR ligands, may play a highly efficient role in amplifying humoral immunity and type 1 polarization. In contrast, agents that induce classical type 2 immune responses, appear to induce minimal DC maturation, at least in vitro. Thus, exosomes derived from such DC might serve to down-regulate immune activation, thus limiting inflammatory sequelae. These data further suggest a potential role for exosomes from mature DC as cell-free vaccines for down-modulating or preventing pathologic type 2 immunity or for boosting Ig responses to poorly immunogenic Ags.

Disclosures
The authors have no financial conflict of interest.

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