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### Mast Cell-Derived Exosomes Induce Phenotypic and Functional Maturation of Dendritic Cells and Elicit Specific Immune Responses In Vivo<sup>1</sup> ✓

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# Mast Cell-Derived Exosomes Induce Phenotypic and Functional Maturation of Dendritic Cells and Elicit Specific Immune Responses In Vivo<sup>1</sup>

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Mast cells (MCs) are considered major players in IgE-mediated allergic responses, but have also recently been recognized as active participants in innate as well as specific immune responses. Recent work provided evidence that MCs are able to activate B and T lymphocytes through the release of vesicles called exosomes. Here we demonstrate that exosomes, which are located in the endocytic pathway, harbor exogenous Ags that associate with other molecules endowed with immunomodulatory functions, including 60- and 70-kDa heat shock proteins. Administration to naive mice of Ag-containing exosomes in the absence of conventional adjuvants elicits specific Ab responses across the MHC II haplotype barrier. We demonstrate that MC-exosomes induce immature dendritic cells (DCs) to up-regulate MHC class II, CD80, CD86, and CD40 molecules and to acquire potent Ag-presenting capacity to T cells. Uptake and processing of Ag-associated exosomes by endogenous DCs were also demonstrated. Finally, exosome-associated heat shock proteins are critical for the acquisition by DCs of the Ag-presenting function. This work demonstrates a heretofore unrecognized collaborative interaction between MCs and DCs leading to the elicitation of specific immune responses. *The Journal of Immunology*, 2003, 170: 3037–3045.

Eukaryotic cells secrete proteins either by the so-called constitutive secretion involving vesicular transport and exocytosis or by the regulated secretion of storage granules upon proper stimulation (1, 2). During exocytosis, in addition to soluble proteins and mediators, vesicles heterogeneous in size and shape, termed exosomes, are released from the lumen of multivesicular bodies in the extracellular environment (3, 4).

Different cell types produce exosomes, including reticulocytes, platelets, B and T lymphocytes, dendritic cells (DCs),<sup>3</sup> macrophages, and intestinal epithelial cells. Recently we have shown that mast cells (MCs) are also a source of exosomes. However, for each cell type, exosomes were described to have distinct and yet not fully understood properties (5). In reticulocytes, secretion of exosomes allows elimination of proteins that are not necessary for the function of differentiated RBC (6). B lymphocyte-derived exosomes bear abundant MHC class II molecules and stimulate T lymphocytes in vitro (7). Recent results have shown that exosomes may be implicated in the transfer of material from one cell to another. For example, MHC class II-containing exosomes of B cell origin are present in abundance on the cell surface of follicular

DCs of human tonsil tissue in vivo (8), which does not express these molecules. Tolerosomes assembled in and released from the small intestinal epithelial cells are another example of exosome-like structures (9). Tolerosomes isolated from serum shortly after Ag feeding or from in vitro-pulsed intestinal epithelial cells are fully capable of inducing Ag-specific tolerance in naive recipient animals. Conversely, exosomes produced by DCs bear not only class II, but also class I, MHC molecules and CD86, an important T cell costimulatory molecule (10). Indeed, tumor peptide-loaded DCs-derived exosomes stimulate strong cytotoxic T lymphocytes that mediate antitumor immune responses and tumor rejection in vivo (10). The mechanism of action of exosomes in vivo is poorly understood. They could either stimulate T and B cells directly through the MHC-peptide complex or may be taken up by professional APCs that, in turn, use peptide-loaded MHC molecules present in exosomes to stimulate T lymphocytes.

MCs are ubiquitously distributed among tissues, including bone marrow and lymphoid tissue. These cells play a central role in allergic responses due to the synthesis of proinflammatory mediators stored in secretory granules. Their ability to phagocytose and process Ags had led different investigators to propose a potential role for MCs in innate immune response against infectious organisms (11). In addition, their capacity to produce a large panel of cytokines (12) suggested that they could influence T and B cell development and activation. We and others have demonstrated that MCs can contribute to the regulation of specific immune responses (13) and that MCs from different species are able to present immunogenic peptides and superantigens to specific T cell clones and T cell hybridomas (14–16). Ag presentation by bone marrow-derived MCs (BMMC) is strictly controlled by cytokines: IL-4 and GM-CSF are potent inducers of the Ag-presenting capacity, whereas IFN- $\gamma$  completely abrogate this function. Thus, MCs synthesize MHC class II molecules, which accumulate in the internal membrane vesicles of multivesicular organelles called secretory granules (4). It has been recently shown that IL-4-treated BMMC

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<sup>3</sup> Abbreviations used in this paper: DC, dendritic cell; BMMC, bone marrow-derived mast cell; hsc, constitutive heat shock protein; hsp, heat shock protein; MC, mast cell; RAP, receptor-associated protein; Tf, transferrin.

and untreated MC lines P815 and MC/9 induce *in vitro* and *in vivo* B and T lymphocyte proliferation and cytokine production (17). This lymphocyte activation was induced by MC-derived factors, since MC supernatants could replace the cell-to-cell contacts (18). We have recently found that the material exerting this immunostimulatory activity is constitutively secreted by MCs and consists of exosomes with a heterogeneous size from 60–100 nm in diameter (17). A characteristic feature of MC-derived exosomes is their potential to induce *in vitro* and *in vivo* B and T lymphocyte activation with IL-2 and IFN- $\gamma$  production and no detectable IL-4. This unique property suggests that MCs may contribute to the development and amplification of specific and nonspecific immune responses.

Based on previous data showing that MC exosomes were located in the endocytic pathway (4), the purpose of this study was to demonstrate whether exogenous Ags associate with exosomes under their native conformation or as processed and to test the potential of Ag-loaded exosomes to prime specific immune responses *in vivo*. Here we demonstrate that endocytosed Ags accumulate in exosomes, and that some internalized Ags are present under both native and processed forms. While seeking a molecular basis for the immunomodulatory function of MC exosomes, we identified the presence of two heat shock proteins, hsp60 and hsc70 (60 and 70 kDa), known for their adjuvant activity on immune responses. We also demonstrate that MC-derived exosomes were highly efficient, in the absence of conventional adjuvants, in inducing specific IgG1 and IgG2a Ab responses *in vivo*. Finally, we provide evidence that MC exosomes are able to induce maturation and functional activation of DCs through cross-presentation of Ag to T cells.

## Materials and Methods

### Mice

DBA/2, C3H/HeJ, and BALB/c (6–8 wk old) were purchased from Janvier (Laval, France). All animal care and experimentation was conducted in accord with the Pasteur Institute animal care and use committee guidelines.

### Reagents and Abs

BSA, transferrin (Tf), and OVA were purchased from Sigma-Aldrich (St. Louis, MO). IFN- $\gamma$  was obtained from Immugenex (Los Angeles, CA). Mouse rIL-3 was purchased from BioSys (Compiègne, France). PE-labeled rat anti-mouse mAbs directed against CD40 (clone 3/23), CD80 (clone RMMP-2), and CD86 (clone RMMP-1) were purchased from Caltag (Burlingame, CA). FITC-labeled anti-mouse Ab directed against CD11c was purchased from BD PharMingen (San Diego, CA). Biotinylated anti-mouse I-A<sup>b/d</sup> mAb was prepared from clone 25-9-17S (American Type Culture Collection, Manassas, VA). PE-labeled anti-mouse IgG2a and FITC-labeled hamster anti-mouse IgG control isotype were purchased from Caltag. Rabbit anti-BSA was purchased from Sigma-Aldrich. Rabbit anti-bovine Tf was purchased from ICN Biochemicals (Costa Mesa, CA). Protein A-agarose beads were purchased from Roche (Mannheim, Germany). Mouse anti-hsp60 (H-1, SC13115) and anti-constitutive hsp70 (anti-hsc70; B-6, SC7298) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse CD4 (clone RL172), CD8 (clone TIB 105), and B cells (B220) were provided by Dr. L. Zitvogel (Institut Gustave Roussy, Villejuif, France). Rabbit anti-hsp70, hsp27, hsp90 $\alpha$ , and hsp90 $\beta$  were provided by Prof. R. M. Tanguay (Laval University, Quebec, Canada). HRP-labeled anti-mouse and anti-rabbit IgG were purchased from DAKO (Copenhagen, Denmark). Anti-CD91 mAb was obtained from BioMac (Leipzig, Germany). The human receptor-associated protein (RAP), the ligand-binding inhibitor of  $\alpha_2$ -macroglobulin receptor/low density lipoprotein receptor-related protein (CD91), was expressed in bacteria as a fusion protein with GST and purified by glutathione-Sepharose. The RAP pGEX expression construct was a gift from Dr. D. Strickland (Holland Laboratory, Rockville, MD).

### Preparation of cells

BMMC were prepared as described by Razin (19) and modified by us (20). After 3 wk of culture using RPMI 1640 (BioWhittaker, Walkersville, MD)

supplemented with 10% heat-inactivated FCS (Roche) and 3 U/ml rIL-3, the cells were harvested and consisted of 98% pure MCs as assessed by toluidine blue staining and c-Kit and Fc $\epsilon$ R1 expression. BMMC were cultured for the last 48 h before harvest in the presence of RPMI complemented with 3 U/ml rIL-3 and FCS depleted of exosomes, allowing the use of supernatant containing only MC-derived exosomes.

DCs were prepared as follows. BMMC suspensions from 7- to 8-wk-old female mice were depleted of lymphocytes with a mixture of anti-mouse CD4, CD8, and B220, and Low-Tox rabbit complement (Cedarlane, Hornby, Canada). After overnight culture at 37°C in complete medium (RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, essential amino acids, and sodium pyruvate), nonadherent cells were recovered and cultured for 5 days in complete RPMI medium with murine rGM-CSF. Murine rGM-CSF produced by the J558 myeloma cell line transfected with the murine GM-CSF gene (provided by Dr. D. Gray) was used at 2% in the culture medium. Cells were harvested on day 5 and used for FACS analysis and *in vitro* assays. On day 5 cells were harvested and stained for FACS analysis and for Ag presentation assays. B cells were prepared as follows. Splenocytes were depleted from T cells with a cocktail of Abs (anti-mouse CD4 and anti-mouse Thy 1) and Low-Tox rabbit complement. The purity of B cells was >95%.

Macrophages were obtained by *in vitro* differentiation of bone marrow precursor cells as described previously (21) in RPMI 1640 supplemented with 10% FCS and 10% fibroblast-conditioned medium (NCTC clone 929). After 5 days of culture, macrophages were detached from bacteriologic plastic petri dishes (Sterilin, Teddington, U.K.) by incubating cells in trypsin solution for 1 min on ice. For induction of MHC class II molecules, macrophages were incubated for 24 h with 25 U/ml murine rIFN- $\gamma$ .

### Induction of *in vitro* bone marrow-derived DC maturation

Day 5 bone marrow DCs were purified with CD11c magnetic beads (Miltenyi, Biotec, Auburn, CA). The purity of CD11c<sup>+</sup> was consistently ~92%. DCs were cultured for an additional 48 h either alone (nonstimulated DCs) or with 1  $\mu$ g/ml MC-, B cell-, or macrophage-derived exosomes or with LPS (1  $\mu$ g/ml) and were double stained with anti-CD11c, anti-MHC class II, anti-CD80, anti-CD86, and anti-CD40 mAbs.

### Immunostaining and flow cytometric analysis

DCs were washed in PBS/1% FCS. After incubation with anti-Fc $\gamma$ RII/RIII Ab (clone 2.4G2; BD PharMingen), cells were incubated with various combinations of the following mAbs: PE-conjugated rat anti-mouse CD40 (3/23), CD80 (RMMP-2), or CD86 (RMMP-1) and biotinylated mouse anti-mouse to I-A<sup>b/d</sup> (clone 25-9-17S) plus streptavidin-PE, and rat FITC-conjugated anti-CD11c. PE-labeled anti-mouse IgG2a and FITC-labeled hamster anti-mouse IgG were used as isotype controls (Caltag). Flow cytometric analysis was performed on a FACScan flow cytometer using the CellQuest software (BD Biosciences, San Jose, CA).

### Exosome purification

Exosomes were prepared from the supernatant of 3-wk-old BMMC cultures as previously reported (17). Three days before exosome collection, BMMC were cultured at  $1 \times 10^6$  cells/ml in IL-3-supplemented medium with 10% FCS depleted of bovine exosomes, and during the last 24 h serum was replaced by ITS (Roche). Supernatants were then subjected to two successive centrifugations at  $300 \times g$  for 5 min and  $1,200 \times g$  for 20 min to eliminate cells and debris, followed by a centrifugation for 2 h at  $70,000 \times g$ . Two fractions were obtained: a high density (pellet) and a low density (hypodense) fraction. Exosomes concentrated in the pellet were washed twice in a large volume of PBS centrifuged at  $70,000 \times g$  for 2 h. The amount of exosomal proteins recovered was measured by Bradford assay (Bio-Rad, Richmond, CA). The same procedure was used for the purification of macrophage- and B cell-derived exosomes.

### Protein analysis by SDS-PAGE, Western blotting, and immunoprecipitation

BMMC ( $5 \times 10^7$ ) were solubilized in lysis buffer containing 25 mM PIPES (pH 7.3), 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 1% Triton X-100, 1 mM sodium orthovanadate (Sigma-Aldrich), 1000 U/ml aprotinin (Sigma-Aldrich), 10  $\mu$ g/ml pepstatin, 20  $\mu$ g/ml leupeptin, and 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (all from Alexis, San Diego, CA). Supernatants were prepared by centrifugation at  $15,000 \times g$  for 30 min. To detect hsp, BMMC lysates and purified exosomes were run on a 12% SDS gel and transferred onto a nitrocellulose membrane. The membrane was saturated with 5%

milk solution and incubated with Abs to constitutive hsp70 (hsc70), inducible hsp70, hsp27, hsp90 $\alpha$ , and hsp90 $\beta$ . To assess the presence of internalized Ags within exosomes, the latter were purified from BMMC ( $10^6$ /ml) preincubated overnight with 100  $\mu$ g/ml Tf, BSA, or OVA. Protein extract from exosomes was subjected to electrophoresis and immunoblotting using rabbit anti-Tf and anti-BSA Ab and mouse anti-OVA Ab. Binding of specific Abs was revealed by a final incubation with HRP-conjugated secondary Abs against mouse and rabbit Igs. Immunoprecipitations were performed with exosomes (100–150  $\mu$ g of total proteins) solubilized in 40  $\mu$ l of PBS/1% Triton X-100 and incubated for 2 h at room temperature with 4  $\mu$ g of anti-Tf Ab or normal rabbit IgG. The Ag-Ab complex was precipitated with 40  $\mu$ l of a 50% slurry of protein A-agarose beads and incubated overnight at 4°C. Beads were recovered by centrifugation for 2 min at 400  $\times$  g and were washed five times with 1 ml of Tris/glycerol buffer (10 mM Tris-HCl (pH 7.6), 50 mM NaCl, 10% (w/v) glycerol, and 0.1% Nonidet P-40). The samples were then incubated at 30°C for 1 h with gentle shaking every 5 min, followed by seven washes with Tris/glycerol buffer. Samples were denatured for 5 min at 90°C with Laemmli loading buffer and were analyzed by SDS-PAGE. Copelleting of hsc70 with Tf was determined by Western blot.

#### *In vitro cross-presentation assay*

Bone marrow-derived DCs ( $5 \times 10^4$ /well) were pulsed with different concentrations of OVA-containing exosomes derived from BMMC, B cells, or macrophages or with exosomes alone and incubated in the presence of the OVA-specific CD4<sup>+</sup> T cell hybridoma 3D0-54.8 ( $5 \times 10^4$ /well) (22). The assay was conducted in a 200- $\mu$ l volume in 96-well plates using RPMI medium containing 10% FCS at 37°C for 24 h. Culture supernatants were harvested and tested for the presence of IL-2.

#### *Assay for Ag presentation by endogenous DCs*

Mice were immunized i.p., intradermally at the tail base, and in the footpad as described in *Results*. CD11c<sup>+</sup> cells were purified as described above from collagenase-digested spleen and lymph node suspensions isolated from immunized mice.

#### *Cytokine detection*

ELISA kits were used for IL-2 and IL-12p70 (R&D Systems, Minneapolis, MN) detection in culture supernatants according to the manufacturer's instructions. To determine the concentration of cytokines in the culture supernatants, standard curves were constructed using recombinant cytokines.

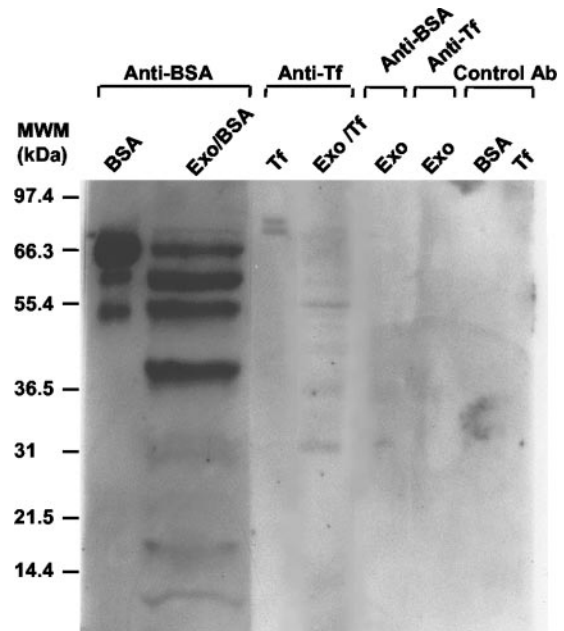
#### *Detection of anti-Tf Abs*

BMMC ( $10^6$ /ml) from DBA/2 mice were incubated for 24 h with 100  $\mu$ g/ml bovine Tf. Exosomes were purified as described above, and the amount of exosomal proteins recovered was measured by Bradford assay. Mice (five mice per group) were immunized s.c. with 5  $\mu$ g of exosomes containing 50 ng of Tf in 0.2 ml of PBS in the absence of conventional adjuvants. Control mice were immunized with 50 ng or 50  $\mu$ g of Tf adsorbed on 2 mg of Al(OH)<sub>3</sub>. Sera were collected 1 wk after a second immunization performed after a 2-wk interval. Tf-specific IgG1 and IgG2a Abs were measured using a solid phase ELISA.

## Results

### *Endocytosed Ags associate with hsp60 and hsc70 within MC exosomes*

Based on ultrastructural features, we have previously demonstrated the presence in MC secretory granules of small vesicles called exosomes (4). These exosomes, which contain mature MHC II molecules and are accessible to endocytic tracers, are therefore positioned along the endocytic route and belong to the late endosome/lysosomal multivesicular bodies (4). To provide biochemical evidence that exosomes are indeed located in the endocytic route, we examined whether exosomes harbor proteins internalized by BMDCs through the fluid phase. As shown in Fig. 1, immunoblot analysis of exosome extract separated by SDS-PAGE demonstrates the presence of bovine Tf and BSA. These results confirm previously reported electron microscopy data showing that Immunogold-labeled BSA was rapidly associated with exosomes after internalization (4). In addition, Tf and BSA were found as native proteins as well as under processed forms, as indicated by the presence of several bands of low and intermediate sizes. While

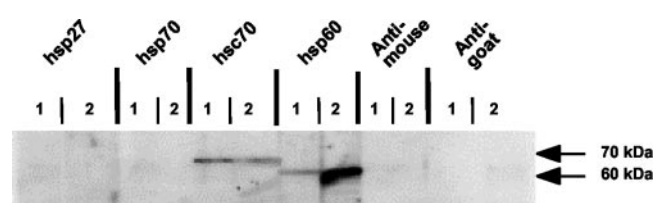


**FIGURE 1.** Exosomes are a site of accumulation of internalized Ags. Exosomes were purified from BMMC incubated with Tf (Exo/Tf) or BSA (Exo/BSA; 100  $\mu$ g/ml) for 24 h. Purified exosomes (15  $\mu$ g) were separated onto 12% SDS-PAGE and immunoblotted with Abs to Tf, BSA, and control Abs. Pure Tf or BSA (1  $\mu$ g) and Ag-free exosomes (Exo) loaded onto the same gel were used as controls. The results are representative of three experiments.

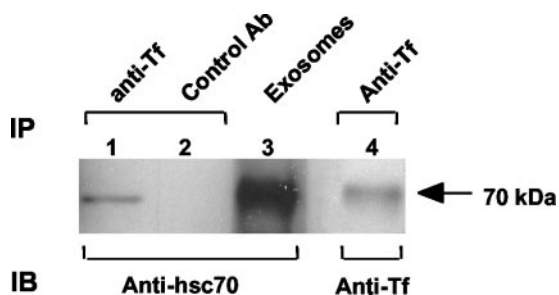
investigating other potential immunologically relevant molecules associated with exosomes, preliminary data using mass spectrometry-based proteomic analysis revealed the presence of a chaperone belonging to the hsp60 family. Here, we extended the study on the presence of other hsps among exosomal proteins and examined by Western blot exosome extracts using a panel of hsp-specific Abs. The data shown in Fig. 2 indicate the presence of hsp60 and hsc70 in both cell lysates and exosomes, but not hsp90, hsp27, or hsp70. Since some hsps are known to act as chaperone molecules, we investigated whether exosome-associated hsps could form complexes with endocytosed exogenous Ags. As indicated in Fig. 3, immunoprecipitates generated by anti-Tf Abs show that hsc70 associated with Tf within exosomes, as revealed by immunoblotting using anti-hsc70 mAb.

### *Ag-associated with exosomes acquire high immunogenicity*

We have previously shown that exosomes are MHC class II-enriched compartments and are the meeting point between these molecules and internalized Ags (4). Furthermore, the same Ags have the ability to associate with chaperone proteins such as hsc70. We

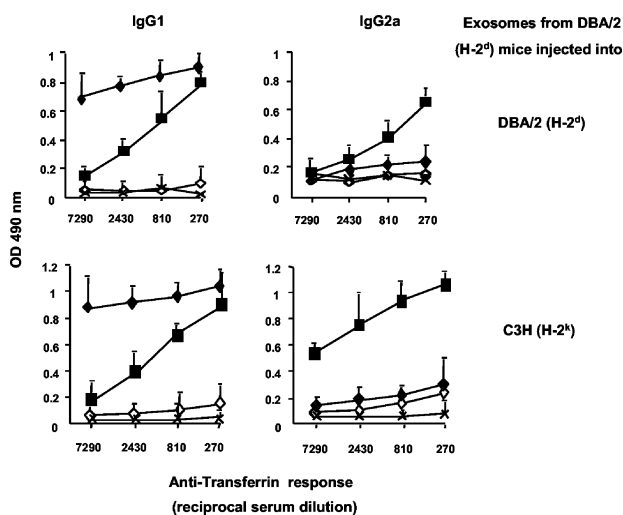


**FIGURE 2.** Presence of hsp60 and hsc70 within exosomes. Purified exosomes (15  $\mu$ g; lane 1) and BMMC lysates (15  $\mu$ g; lane 2) were loaded onto a 12% SDS-PAGE, transferred to a nitrocellulose sheet, and blotted with Abs against hsp27, hsp70, hsp60, and hsc70 and with control Abs. The results are representative of six experiments.



**FIGURE 3.** Tf is present in a complexed form with hsc70 within exosomes. Protein extracts from BMMC-derived exosomes were immunoprecipitated (IP) with anti-Tf Ab (lanes 1 and 4) or with control Ab (lane 2), resolved onto a 12% SDS-PAGE, and immunoblotted (IB) with anti-hsc70 (lanes 1–3) or with anti-Tf Ab (lane 4). As a positive control, exosomes were loaded onto the gel and immunoblotted with anti hsc70 Ab (lane 3). The arrow indicates a specific band corresponding to the expected m.w. for hsc70 and Tf. The experiment was reproduced twice with similar results.

made the assumption that such exosomes may constitute a potent cell-free system for Ag delivery to trigger Ag-specific responses in vivo. To this aim, DBA/2 mice were immunized with exosomes isolated from syngeneic BMMC, which have been loaded with Tf or BSA. The amount of Ag associated with exosomes was determined by running side-by-side on an electrophoretic gel a given quantity of exosomes and a range of known concentrations of the purified Ags. As an average from four separate experiments, we could estimate the proportion of BSA or Tf associated with exosomes to be ~1% of total exosomal proteins (data not shown). We examined the immunogenic potential of bovine Tf associated with exosomes compared with the capacity of the same Ag administered via alternative vehicles to induce specific immune responses. Fig. 4 shows that exosome-associated Tf (50 ng for 5  $\mu$ g of exosomes) was extremely efficient in eliciting both IgG1 and IgG2a



**FIGURE 4.** Elicitation of specific Ab responses in mice immunized with exosome-associated Ags. Exosomes purified from BMMC (DBA/2 mice) cultured for 24 h with Tf (100  $\mu$ g/ml) were injected s.c. into syngeneic DBA/2 or allogeneic C3H mice. The actual amount of Tf associated with exosomes injected was 50 ng (■). Control mice were injected with 50 ng of Tf in PBS (X) or adsorbed onto 2 mg of Al(OH)<sub>3</sub> (◇) or with 5  $\mu$ g of Tf adsorbed onto Al(OH)<sub>3</sub> (◆). Sera were collected 1 wk after a series of two immunizations performed at 2-wk intervals, and Tf-specific IgG1 and IgG2a Abs were measured by ELISA. The results are representative of four experiments, and the values shown represent the means from duplicate wells.

Abs, whereas no Ab response could be observed when the same dose of Tf (50 ng) was administered in the presence of PBS or adsorbed on alum.

To investigate whether the immunostimulatory potential of exosomes is related to the presence of MHC II molecules, we performed cross-priming experiments in which exosome-associated Tf from H-2<sup>d</sup> mice were injected into either syngeneic H-2<sup>d</sup> DBA/2 mice or allogeneic H-2<sup>k</sup> C3H mice (Fig. 4). The data show that C3H mice respond equally and even better than DBA/2 mice, suggesting that anti-Tf Ab responses could be elicited across the MHC haplotype barrier. These findings indicate that other exosome-associated components provide their immunostimulatory or adjuvant activity in an MHC II-independent manner.

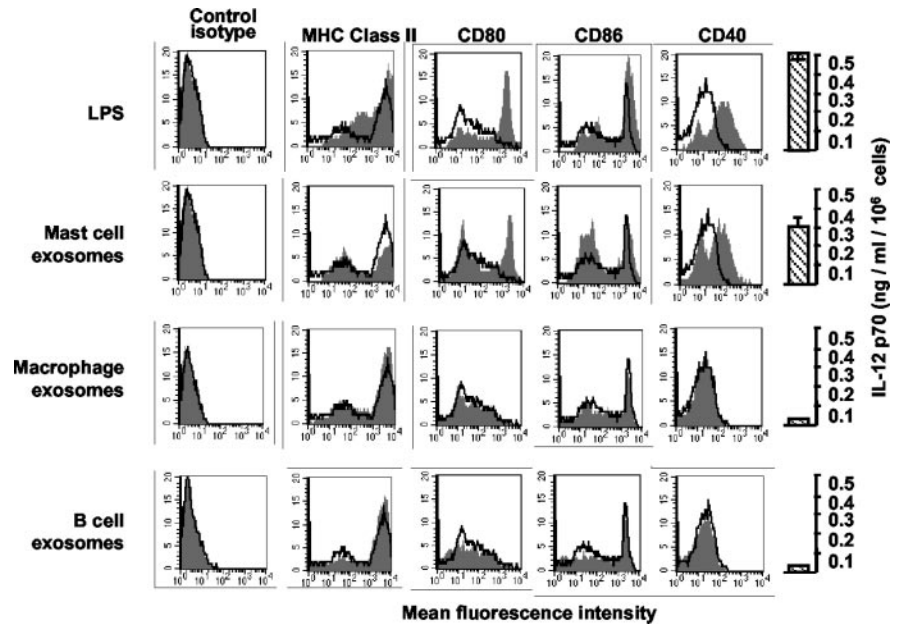
#### DC maturation induced by MC-derived exosomes

Since exosomes were able to elicit Ag-specific immune responses in an MHC-independent manner, we postulated that exosomes deliver Ags to professional APCs through cross-presentation. DCs are known as the most effective APCs in the initiation of specific immune responses by stimulating naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells. However, resting DCs require external stimuli to undergo a maturation process, after which they acquire full expression of their function. Using bone marrow-derived DCs, we analyzed the phenotypic alterations occurring upon exposure to exosomes obtained from various cell origins. DCs were incubated with LPS as a positive control or in the presence of exosomes derived from BMMC, bone marrow-derived macrophages, or purified splenic B lymphocytes. After 48 h of incubation, cell surface expression of MHC II, CD80, CD86, and CD40 was measured by FACS. As shown in Fig. 5, in contrast to exosomes derived from macrophages and B lymphocytes, which had no effect, MC-derived exosomes strongly up-regulated the expression of all tested markers. The most striking effect was observed for CD80 and CD40. In parallel, IL-12p70 was found in the supernatants from DCs activated by MC-derived exosomes and LPS, but not from those obtained with B cell- and macrophage-derived exosomes. These data demonstrate that exosomes from MCs induce phenotypic and functional maturation of DCs, and this may represent a mechanism by which exosomes efficiently prime the immune system in vivo.

#### Selective enrichment of hsp60 and hsc70 in exosomes from BMMC

Combining data from the in vivo immune response induced by MHC class II-mismatched exosomes (Fig. 4) and the unique ability of BMMC-derived exosomes compared with exosomes from B cells and macrophages to induce DC maturation (Fig. 5), we estimated the relative amounts of hsp60 and hsc70 present in exosomes isolated from these different cell types. Similar amounts of exosomes and cell lysates (5  $\mu$ g) from B cells, BMMC, and macrophages were loaded onto a 12% SDS gel, and the presence of hsp60 and hsc70 was quantified by Western blot. As shown in Fig. 6A, hsp60 was highly enriched in BMMC-derived exosomes, whereas no detectable or only a limited amount was present in exosomes from B cells and macrophages, respectively. A similar pattern was observed for hsc70 (Fig. 6B), but in contrast to hsp60, this protein was present at a very low level in both B cell- and macrophage-derived exosomes. These data demonstrate that hsp60 and hsc70 are selectively enriched in exosomes from BMMC, but not in those isolated from B cells and macrophages, providing a possible molecular mechanism by which these particular exosomes affect DC maturation and the Ag-specific immune response.

**FIGURE 5.** Phenotypic and functional DC maturation induced by MC-derived exosomes. Immature bone marrow-derived DCs on day 5 of culture were purified using the MACS column system. CD11c<sup>+</sup> cells were incubated at 37°C either alone (nonstimulated DCs) or with 1 μg of MC (BMMC)-, B cell-, and macrophage-derived exosomes. Positive and negative controls for DC maturation were used (LPS (1 μg/ml) and PBS). After 48 h of incubation, the cells were collected and stained for the DC-specific marker CD11c and the activation markers MHC class II, CD80, CD86, and CD40. The percentage and mean fluorescence intensity were analyzed by FACS. In parallel, supernatants were tested for their content of IL-12p70, as measured by ELISA. The results are representative of three experiments with similar results.



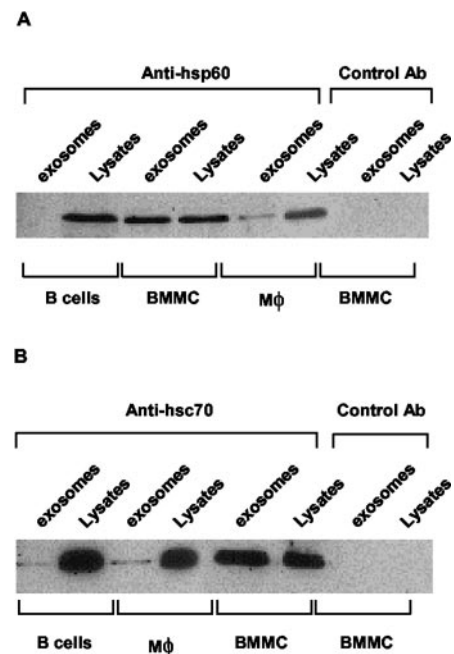
*MC-derived exosomes induce DCs to become efficient APCs*

To evaluate the efficacy of exosome-associated OVA compared with soluble OVA to provide DCs with APC function, we first assessed the actual amount of OVA present within exosomes. Fig. 7A shows semiquantitative analysis by SDS-PAGE of exosome-associated OVA compared with various amounts of purified OVA, followed by immunoblotting with anti-OVA-specific Abs. The amount of total exosomal proteins loaded onto the gel was 8 μg, and according to the band intensity of OVA contained in exosomes (*right* band), we could estimate the amount of OVA to be ~0.12 μg, which represents 1.5% of the total exosomal proteins.

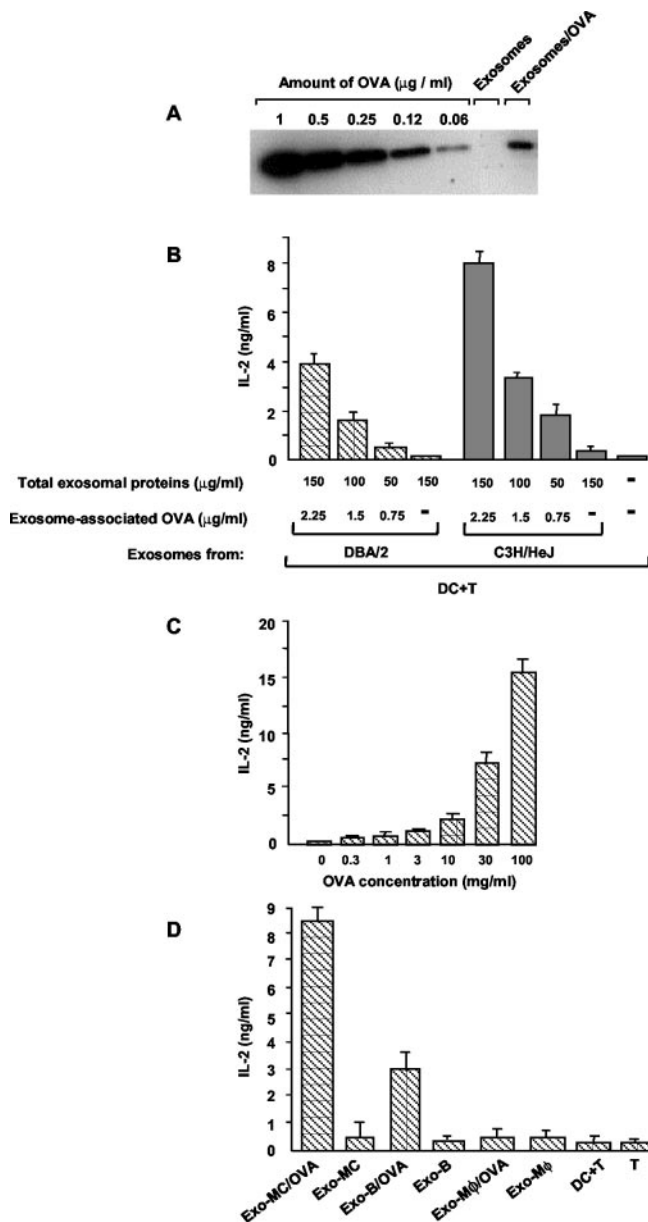
To demonstrate whether exosome-associated Ags can be transferred to and presented by MHC class II molecules on DCs, we cocultured bone marrow-derived DCs from DBA/2 mice with exosomes alone or with various doses of OVA-containing exosomes from syngeneic DBA/2 (H-2<sup>d</sup>) or allogeneic C3H/HeJ (H-2<sup>k</sup>) mice, and tested their capacity to stimulate the H-2<sup>d</sup>-restricted OVA-specific T cell hybridoma 3DO-54.8. As shown in Fig. 7B, dose-dependent IL-2 production by the 3DO-54.8 T cell hybridoma could be induced by OVA-containing exosomes, whereas control exosomes or OVA-containing exosomes in the absence of DCs (data not shown) failed to activate T cells. Interestingly, exosomes from both DBA/2 and C3H/HeJ BMMC could be presented by DCs from DBA/2 mice, indicating that Ag associated with MHC class II-mismatched exosomes can be cross-presented by DCs to T cells.

We compared the efficiency of exosome-associated OVA and soluble native OVA to be presented by DCs to a OVA-specific T cell hybridoma. As shown in Fig. 7C, a dose-dependent IL-2 production was induced with a detection limit at 1 μg/ml OVA. According to the standard curve of IL-2 response provided by soluble OVA, DCs loaded with OVA associated with exosomes (Fig. 7B) were much more efficient in stimulating T cells. A quantitative estimation indicates that the potency of exosome-associated OVA to elicit T cell responses was ~18-fold (BMMC exosomes from DBA/2 mice) and 43-fold (BMMC exosomes from C3H/HeJ mice) higher than that of soluble OVA. These data fit well with the high efficiency of Ag-containing exosomes in inducing Ag-specific Abs in vivo. To verify whether the inability of exosomes from B cells and macrophages to induce DC maturation (Fig. 5) correlates with

their lack of T cell stimulatory potential, exosomes from different cell types were tested for their capacity to provide Ag-presenting capacity to naive DCs. Fig. 7D represents the IL-2 response of OVA-specific T cell hybridoma to the highest dose of exosome-associated OVA presented by DCs. Only exosomes from MCs and at a much lower degree those from B cells were able to induce DCs to activate T cells.



**FIGURE 6.** Comparative analysis of the hsp60 and hsc70 contents in exosomes from different cell types. Lysates (5 μg) and purified exosomes (5 μg) from BMMCs, macrophages (Mφ), and B cells were loaded on a 12% SDS gel and immunoblotted with Abs to hsp60 (A) and hsc70 (B) and with isotype-matched mouse control Ab. The experiment was reproduced twice with similar results.



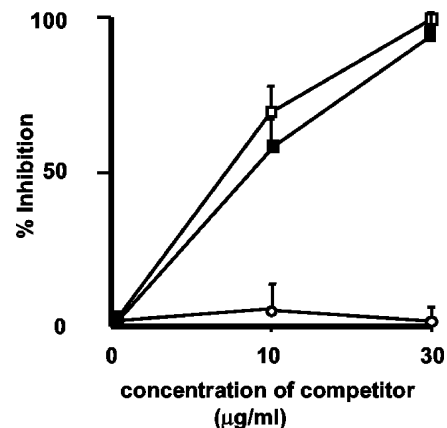
**FIGURE 7.** Induction of Ag-presenting function of DCs by MC-derived exosomes. *A*, Exosomes were purified from DBA/2 and C3H/HeJ mouse BMMCs incubated overnight with or without OVA (100  $\mu\text{g/ml}$ ). Purified exosomes (8  $\mu\text{g}$ ) were separated by SDS-PAGE (12%) and immunoblotted with Abs to OVA. Different concentrations of pure OVA were loaded on the same gel and used as a reference to titrate OVA associated with exosomes. Similar amounts of OVA were recovered from both mouse strain BMMC exosomes, and for simplification only exosomes from DBA/2 were analyzed. *B*, Exosomes were purified from DBA/2 and C3H/HeJ mouse BMMC pulsed, or not, overnight with 100  $\mu\text{g/ml}$  OVA. Different mouse concentrations of exosomes containing OVA, or not, were cultured with immature bone marrow DCs from DBA/2 mice ( $5 \times 10^4/\text{well}$ ) in the presence of the H-2<sup>d</sup>-restricted OVA-specific T cell hybridoma 3DO-54.8 ( $5 \times 10^4/\text{well}$ ). *C*, The T cell hybridoma 3DO-54.8 was cultured alone or with DCs in the absence or the presence of different concentrations of OVA. *D*, Exosomes alone or exosome-associated OVA from MCs, B cells, and macrophages were added at various concentrations to a coculture of DCs and OVA-specific T cell hybridoma cells. For simplification, only the IL-2 response corresponding to the highest dose of exosomes (150  $\mu\text{g/ml}$ ) is represented. *B–D*, IL-2 was measured in the supernatants after 24 h of culture. The experiment was repeated twice with similar results.

### Presentation of exosome-associated Ag by DCs requires functional CD91 receptor

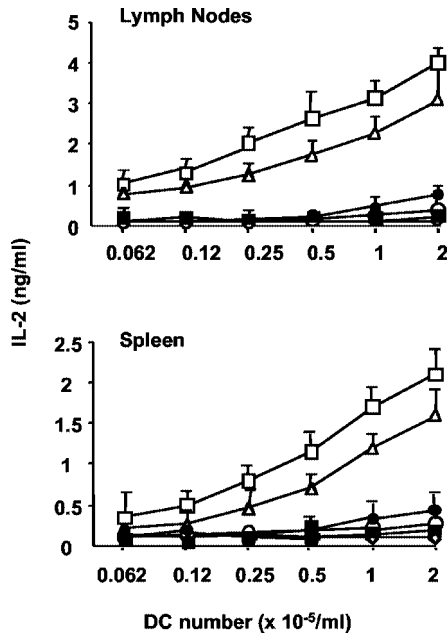
To explore the molecular basis of the remarkable immunogenicity of exosome-associated Ags and to provide explanation for the direct activation and maturation of DCs by exosomes, we set up experiments to investigate the role of hsp in this process. CD91 has been identified as the common receptor for hsp, including gp96, hsp90, hsp70, calreticulin, hsp60, and  $\alpha_2$ -macroglobulin (23, 24). On the other hand, CD91 is an endocytic receptor that internalizes antigenic proteins or peptides chaperoned by hsp (23). This CD91-mediated Ag endocytosis provides APCs with strong T cell stimulatory activity. CD91 receptors on DCs were blocked by anti-CD91 Abs, control Abs, or RAP, a ligand of CD91 receptor (25), before the cells were added to OVA-specific T cell hybridoma 3DO-54.8 together with exosomes isolated from BMMCs loaded with OVA. As shown in Fig. 8, IL-2 production was specifically inhibited by either anti-CD91 Ab or RAP in a dose-dependent manner. This result suggests that cross-presentation of OVA by DCs operates via CD91-mediated internalization of exosome-associated hsc70- or hsc60-OVA complexes.

### Uptake by DCs of Ag-loaded MC exosomes delivered in vivo

To test whether MC-derived exosomes influence the maturation and the function of DCs in vivo, mice were injected with exosomes containing OVA or not, with OVA in the presence of LPS, or with PBS. Since Ag was simultaneously administered through different routes, namely at the tail base, in the footpads, and i.p., the Ag-presenting capacity of DCs from both spleen and lymph nodes was tested. Lymph node and spleen cells were harvested 14 h after Ag delivery, and DCs were purified on anti-CD11c-coated beads. Functional presentation of OVA by DCs was assessed by the ability of these cells to induce IL-2 production by the OVA-specific T cell hybridoma 3DO-54.8. As shown in Fig. 9, DCs from mice injected with OVA-containing exosomes (150 ng of OVA/mouse) derived from MCs, but not those from mice treated with OVA-containing exosomes from B cells and macrophages, were able to specifically induce IL-2 production by OVA-specific T cell hybridoma. The efficiency of the in vivo-induced Ag-presenting capacity of MC-derived exosomes by DCs was demonstrated by the



**FIGURE 8.** Uptake and Ag presentation of exosomes by DCs require functional CD91 receptor. BM-DCs ( $5 \times 10^4/\text{well}$ ) were preincubated for 30 min with different concentrations of CD91 Ab ( $\square$ ), an isotype-matched control Ab ( $\circ$ ), and RAP ( $\blacksquare$ ), a ligand of the CD91 receptor. Then, exosomes purified from BMMC pulsed, or not, overnight with 100  $\mu\text{g/ml}$  OVA were added to the culture, followed by addition of OVA-specific T cell hybridoma 3DO-54.8 ( $5 \times 10^4/\text{well}$ ). After 24 h of culture, IL-2 production was determined. The data are representative of three independent experiments, and the values shown represent the mean from duplicate wells.



**FIGURE 9.** Uptake of exosome-associated Ag by DCs in vivo. DCs were purified from lymph nodes and spleen of DBA/2 mice using the MACS column system 14 h after injection of OVA (150 ng)-containing exosomes, isolated from BMMC ( $\Delta$ ), B lymphocytes ( $\bullet$ ), and macrophages ( $\blacksquare$ ) pulsed overnight with OVA (100  $\mu$ g/ml). Control groups consisted of mice injected with 150 ng of OVA in the presence of 5  $\mu$ g of LPS ( $\square$ ) or with PBS ( $\circ$ ). Additional controls consisting of mice injected with OVA-free exosomes from the three cell types induced no significant IL-2 responses ( $<10$  pg/ml) and are not represented in the figure. T cells were also cultured alone ( $\diamond$ ). Different concentrations of CD11c<sup>+</sup> cells were cultured with OVA-specific T cell hybridoma 3DO54.8 ( $5 \times 10^4$ /well). The data are representative of three independent experiments.

similar magnitude of T cell stimulatory capacity as that induced by delivering 150 ng of OVA in the presence of 5  $\mu$ g of LPS. The same dose of OVA administered in the presence of PBS did not result in Ag presentation by DCs. Furthermore, the IL-2 response induced by DCs from spleen was about half that obtained from lymph node DCs. These data indicate that MC exosomes constitute a powerful Ag delivery device that can arm endogenous DCs for efficient Ag presentation.

## Discussion

The aim of this study was to investigate the mechanisms by which MCs can modulate immune responses through the release of exosomes. Our results indicate that MC-derived exosomes harbor exogenous Ags as well as endogenous hsps and, once delivered into naive mice, give rise to potent Ag-specific Ab responses. More importantly, exosomes are able to induce phenotypic as well as functional maturation of DCs in vitro and in vivo.

Exosomes have now been described in several cell types, including B lymphocytes, DCs, reticulocytes, platelets, MCs (5), and, more recently, epithelial cells (26), and their function apparently differs from one cell type to another. With regard to MCs, we have recently demonstrated the ability of MC exosomes to induce Ag-independent B and T lymphocyte activation in vitro and in vivo (17). To extend these findings we asked whether this so-called adjuvant property of exosomes would also support Ag-specific immune responses, and what the molecular basis would be for such activities. Indeed, in previous studies, we reported using electron microscopy in the presence of MHC II and costimulatory molecules (4, 17) within exosomes and found that these MHC II-rich

compartments are rapidly accessed by exogenous Ags after a time lag of 20 min (4). We here confirm that MC exosomes are connected to the endocytic pathway, since we found the presence within these compartments of at least two exogenous Ags, bovine Tf and BSA. In addition, we show the presence within exosomes of two hsps, hsp60 and hsc70, a constitutively expressed member of the hsp70 family. As a chaperone molecule, hsc70 is able to bind proteins and peptides, and it has been proposed that by this mean bound epitopes are protected from extensive proteolytic degradation. Although a majority of reports indicate that the chaperone activity of hsp70 family members facilitate the loading of MHC I molecules with peptides (27, 28), it is now established that constitutively expressed hsc70 is present in lysosomal compartments (29) and facilitates MHC II Ag processing and presentation (30, 31). Accordingly, it has been previously shown that Abs against hsp70 family members as well as the hsc70-binding compound deoxyspergualin inhibit MHC II Ag processing and presentation (32). On the other hand, efficient generation of immunogenic peptides and subsequent effective Ag presentation occur through a mechanism by which hsp-bound peptides are rapidly transferred to the MHC II peptide groove that becomes vacant upon catalytic activation of the H2-M molecule (33).

The colocalization within exosomes of exogenous Ags and hsc70 led us to explore whether, as a chaperone molecule, hsc70 would form complexes with these Ags. Using immunoprecipitation procedures we indeed found that complexes between hsc70 and Tf are present within exosomes. Moreover, administration of Tf-containing as well as BSA-containing exosomes to mice induced a strong production of Ag-specific Abs in the absence of conventional adjuvants. More strikingly, IgG1 and IgG2a Ab responses to Tf were elicited not only in syngeneic (H-2<sup>d</sup>), but also in allogeneic (H-2<sup>k</sup>), mice. These results support the hypothesis that exosomes not only display antigenic peptides in the context of donor MHC II molecules, but also harbor exogenous Ags that can be transferred to host APCs, a mechanism termed cross-priming (34), and induce an Ag-specific immune response. The hsc70/Ag complexes were likely candidates for such a mechanism. Several reports have demonstrated that hsps are specialized carriers of antigenic peptides in vitro and in vivo and that they play a role in tumor immunogenicity. The proposed mechanisms by which hsps enhance immune responses against tumor Ags are 1) they constitute an immunological danger signal (35); 2) once released from dying cells they serve as vehicle that transfers peptides to professional APCs (36); and 3) in living tumor cells, direct Ag presentation to tumor-specific T cells is enhanced by hsps (37). Indeed, hsps, like various signals, including cytokines IL-1 $\beta$  and TNF- $\alpha$  (38), bacterial products such as LPS, and other micro-organisms, are able to induce DC maturation. Whereas immature DCs are very effective in Ag capture (34), mature DCs are more potent activators of naive T cells (38–43) and thus initiate the adaptive immune response. In agreement with this view, MC-derived, hsp-containing exosomes act as direct inducers of DC maturation, and this could explain both the mitogenic activity of exosomes (18) and the high immunogenic potential of exosome-associated Ags reported here. Interestingly, this maturation effect on DCs is unique to MC-derived exosomes, since those from B cells and macrophages were not able to convert DCs to the mature phenotype. One possible explanation for this differential effect is that exosomes from different cell types (MCs, DCs, and B cells) might accumulate variable amounts of biologically active molecules. It is indeed the case for hsps, since equivalent amounts of hsp60 and hsc70 could be detected by Western blot in exosomes and in whole cell lysates from MCs, whereas, in contrast, the two hsps were virtually absent



from exosomes derived from B cells and macrophages. Enrichment of hsp60 and hsc70 in MC-derived exosomes may constitute a molecular mechanism by which these particular exosomes become efficient in triggering Ag-specific immune responses.

To establish a direct relationship between exosomes, hsp, and DC activation, we explored the role of CD91, an endocytosing receptor that binds  $\alpha_2$ -macroglobulin, hsp70, hsp90, gp96, calreticulin, and perhaps other hsp (23, 24). Our experimental design was based on the uptake and Ag presentation of exosome-associated OVA to OVA<sub>323-339</sub>-specific T cell hybridoma in the absence or presence of anti-CD91 mAb or with RAP, a CD91-binding protein (25, 44). The dramatic specific blockade of T cell activation that occurred when anti-CD91 mAb or RAP was added to the cultures demonstrates that the uptake and/or presentation of exosome-associated OVA by DCs require accessible and functional CD91 receptors. As a cytosolic protein, hsc70 should be present only in the lumen of exosomes (45); however, it has been reported that it can accumulate on the surface of exosomes present in multivesicular bodies of macrophage and DC lines (46), explaining how the CD91 receptor of DCs in our experiments has access to it.

Evidence of efficient uptake of exosome-associated Ags by DCs in vivo was provided by the ability of spleen- and lymph node-derived DCs obtained from mice primed with OVA-containing exosomes to activate OVA-specific T cells in vitro. Given the high immunogenic potential of Ags associated with exosomes, this can be taken as an original cell-free system that can be used as a vaccine, particularly for peptides present at low frequency or Ags endowed with weak immunogenicity. This adjuvant activity gives rise to enhanced Ab responses, as documented in the present work, but could also generate MHC class I-restricted CD8<sup>+</sup> T cell responses. Indeed, it has been reported that mouse DCs release exosomes displaying peptide-loaded MHC class I complexes. Interestingly, when these exosomes were loaded with tumor-derived peptides, they were able to elicit CTL responses and eradicate established murine tumors (10).

MCs (47) and DCs (48) are sentinels of the innate immune system that play a significant role in acquired immunity. Since MCs colocalize with immature DCs and can be rapidly and directly activated by pathogens or Ags, we postulate that the MC-dependent inflammatory reaction will have a major impact on the function of DCs. We view the mechanisms described in the present work as part of a sentinel function fulfilled by MCs and DCs to mount Ag-specific immune responses. Although as yet no direct evidence exists in vivo, it is conceivable that cross-presentation by DCs of exosome-associated Ag derived from MCs may represent an amplification mechanism by which priming of naive T cells with minute amounts of Ags may occur.

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