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_J Immunol_ 2004; 172:2126-2136; doi: 10.4049/jimmunol.172.4.2126
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Exosomes as Potent Cell-Free Peptide-Based Vaccine. I. Dendritic Cell-Derived Exosomes Transfer Functional MHC Class I/Peptide Complexes to Dendritic Cells

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Current immunization protocols in cancer patients involve CTL-defined tumor peptides. Mature dendritic cells (DC) are the most potent APCs for the priming of naive CD8+ T cells, eventually leading to tumor eradication. Because DC can secrete MHC class I-bearing exosomes, we addressed whether exosomes pulsed with synthetic peptides could subserve the DC function consisting in MHC class I-restricted, peptide-specific CTL priming in vitro and in vivo. The priming of CTL restricted by HLA-A2 molecules and specific for melanoma peptides was performed: 1) using in vitro stimulations of total blood lymphocytes with autologous DC pulsed with GMP-manufactured autologous exosomes in a series of normal volunteers; 2) in HLA-A2 transgenic mice (HHD2) using exosomes harboring functional HLA-A2/Mart1 peptide complexes. In this study, we show that: 1) DC release abundant MHC class I/peptide complexes transferred within exosomes to other naive DC for efficient CD8+ T cell priming in vitro; 2) exosomes require nature’s adjuvants (mature DC) to efficiently promote the differentiation of melanoma-specific effector T lymphocytes producing IFN-γ (Tc1) effector lymphocytes in HLA-A2 transgenic mice (HHD2). These data imply that exosomes might be a transfer mechanism of functional MHC class I/peptide complexes to DC for efficient CTL activation in vivo. The Journal of Immunology, 2004, 172: 2126–2136.

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Received for publication May 9, 2003. Accepted for publication November 25, 2003.

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1 This work (and N.E.C.S.) was supported by European Community Grant QLRT-2001-00093, the LIGUE française contre le Cancer, the Association pour la Recherche sur le cancer, and the Ministry of Luxembourg (to N.E.C.S.). F.A. was supported by a Young Investigator Award from the American Society of Clinical Oncology. N.C. was supported by a European Fellowship in the QLRT-2001-00093. N.E.C.S. was supported by the Ministère de la Culture, de l’Enseignement supérieur et de la Recherche du Luxembourg, and by the Fondation Luxembourgoise Contre le Cancer. This work was also supported by Anosys, Inc.


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4 Abbreviations used in this paper: DC, dendritic cell; BM-DC, bone marrow-derived DC; Ex, DC-derived exosome; FDC, follicular DC; iDC, immature DC; LN, lymph node; MD-DC, monocyte-derived DC; mDC, mature DC; NV, normal volunteer; Tc1, effector T lymphocytes producing IFN-γ; ELA, Mart116–18 peptide.
optimal CTL priming; and 2) exosomal MHC class I molecules substitute for those expressed on plasma membrane of mature DC (mDC) for the initiation of synthetic peptide-specific, MHC class I-restricted CD8+ T cell responses in vivo. Indeed, priming of Mart1-specific CD8+ T cells in HLA-A2 transgenic mice using 2 × 109 HLA-A2+/Mart1-bearing exosomes was as efficient as 3 × 109 mDC loaded with saturating μM ranges of Mart1 peptides. Because 2 × 109 exosomal MHC class I molecules can be released by 10^7–10^8 DC in vitro and because exosomes require transfer onto DC to activate CD8+ T cells, exosomes can be viewed as an amplification process for DC-mediated CTL responses.

Materials and Methods

DC culture

Monocyte-derived DC (MD-DC) were generated from purified monocytes in bags (Nexell, Brussels, Belgium) under adherence-free conditions, as previously described (21). DC were differentiated from monocytes cultured in AIMV supplemented with 1000 IU/ml human rGM-CSF and 700 IU/ml human rIL-4 (Novartis, Norderstedt, Germany). After 4 days, maturation was induced by adding LPS (5 μg/ml; Sigma-Aldrich) to culture medium at 37°C, followed by three washing steps in saline buffer. Adherent cells were propagated in AIMV complete medium containing 1000 IU/ml of both human rGM-CSF and human rIL-4 (Novartis and Schering-Plough, respectively). For in vitro testing, DC were used at day 6. Maturation was induced by LPS (1 μg/ml; Sigma-Aldrich, St. Quentin Fallavier, France) for 24 h. PBMCs and MD-DCs were assayed by flow cytometry analysis with MA2.1 Ab-containing ascites, CD14 (BD Biosciences, Heidelberg, Germany), CD1a (BD Pharmingen, Heidelberg, Germany), CD83 (BD Pharmingen), HLA-DR (BD Pharmingen), and CD40 (BD Pharmingen).

Mouse bone marrow-derived DC (BM-DC) were cultured, as previously described (12). Briefly, bone marrow progenitor cells were grown in IMDM culture medium (Sigma-Aldrich) supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM l-glutamine, 1 mM Na pyruvate (Life Technologies), and 10% decomplemented FCS (Pan Biotech, Aidenbach, Germany). Bulk lymphocytes were depleted by a 3-h adherence at 37°C, followed by three washing steps in saline buffer. Adherent cells were propagated in AIMV complete medium containing 1000 IU/ml of both human rGM-CSF and human rIL-4 (Novartis and Schering-Plough, respectively). For in vitro testing, DC were used at day 6. Maturation was induced by adding LPS (5 μg/ml; Sigma-Aldrich) to culture medium for 24 h. For in vivo immunization protocols, BM-DC were used at day 12. The phenotype of BM-DC was analyzed by flow cytometry using anti-mouse CD11c, I-Ak, CD80, CD86, and CD40 mAb (BD Pharmingen), and H-2Kd and H-2Dd (22). In addition, BM-DC propagated from HHD2 mice were stained with MA2.1 Ab-containing ascites.

Cell lines

Melanoma tumor cells (FON, HLA-A2+/Mart1+/14)) were expanded in RPMI 1640 (Life Technologies) supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM l-glutamine, 1 mM Na pyruvate (Life Technologies), and 10% decomplemented FCS (Sermoed).

Exosomes production and purification

Exosomes were either isolated using the classical process of a series of differential centrifugations, already described (11, 12) or isolated according to a process of ultracentrifugation/dialfiltration derived from Lamparski et al. (23). Briefly, 2–4 L of DC culture medium was microfritrated (3 μm/0.8 μm) and then ultrafiltrated through a 500-kDa filter up to a final volume of 50 ml. This 50 ml of exosome-containing medium was supplemented with up to 1 L of PBS, and a second step of 500-kDa ultrafiltration was performed, leading to a final volume of 20–50 ml. This preparation was ultracentrifuged at 100,000 g onto a D20/30% sucrose gradient-density cushion (d = 1.217). The exosomal pellet recovered in the cushion was dialylated for sterilization and will be referred to as exosomes henceforth.

MHC class I immunocapture assays for exosomal MHC class I quantification

A titrated mouse anti-human MHC class I mAb (HLA-A, B, C; BD Pharmingen) was incubated with excess exosomes that had dried in a 96-well plate and subsequently blocked with 6% nonfat milk. After a 1-h incubation, the plate was washed and incubated with addition of excess goat anti-mouse Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) conjugated to HRP for 1 h at room temperature. The plate was developed for 5 min using the ECL substrate system, as described by the manufacturer (Amersham Pharmacia, Saclay, France). Chemiluminescence signal was measured by the Wallac trilux chemiluminometer (PerkinElmer, Gaithersburg, MD).

Pulsing of exosomal MHC class I peptide complexes with Mart126–35 peptides

To elute the endogenous MHC class I peptides bound to exosomes, 100 μl of exosomes (~10^7 MHC class I molecules) is treated with an equal volume of acetate buffer, pH 5.1, containing the synthetic CTL epitope MelanA/Mart1<sub>ELAGIGILTV</sub>, at 10 μM at 4°C for 30 min (ExMart1). After such an extraction, the preparation is neutralized with a Tris buffer, pH 11, on ice for 15 min to allow reformation of the trimeric MHC class I peptide complexes. Then unbound peptides and debris are removed using an ultracentrifugation (100,000 × g/min, 40 min) step on a D20/30% sucrose gradient-density cushion. The exosomes recovered in the cushion are subsequently ultracentrifuged (100,000 × g, 1 h). The pellet is resuspended in PBS once again and stored at −80°C. Unbound peptides cannot exceed a final concentration of 1–7 nM.

In vitro assays using exosomes for CTL activation

LT11, a Mart1<sub>26–35</sub>—specific HLA-A2-restricted BV9 CD8+ T cell clone, has been previously described (14). A total of 2 × 10^6 DC was pulsed for 2 h with increasing amounts of exosomes (from 10^3 to 10^5 MHC class I molecules) in 30 μl of culture medium at 37°C before incubation with 2 × 10^4 LT11 in a final volume of 200 μl. DCTLT11 cultures were performed in round-bottom 96-well plates in RPMI complete medium supplemented with 10% human pooled AB serum. Positive control HLA-A2+ DC were pulsed with 0.1–10 μg/ml of Mart1<sub>26–35</sub> and washed before incubation with LT11. Twenty-four-hour supernatants were assayed in IFN-γ ELISA (Immunotech, Marseille, France).

In vitro stimulation of PBL with exosomes

A total of 2 × 10^6 T2 cells or 3 × 10^6 HLA-A2+ MD-DC was incubated with exosomes (10^9 and 2 × 10^10 MHC class I molecules/well, respectively, HLA-A2+ or HLA-A2−) loaded with MelanA/Mart1 at 37°C in 50 μl. After a 2-h incubation, PBL from healthy volunteers were added to T2 cells at a PBL-T2 ratio of 10:1, or to MD-DC at a PBL-DC ratio of 3:1. In the latter setting, all three components (i.e., MD-DC, exosomes, and PBL) were autologous, except for negative controls (HLA-A2− exosomes pulsed onto HLA-A2+ DC). Cultures were performed in round-bottom 96-well plates in RPMI complete medium supplemented with 10% human pooled AB, 100 U/ml IL-2 (Proleneuk; Chiron, Emeryville, CA), and T cell growth factors (14). Two or three weekly stimulations of PBL were performed. Positive controls included MD-DC pulsed with 10 μM Mart1<sub>26–35</sub>. On days 7, 14, and 21, the proportion of Mart1-specific CD8+ T cells was assessed in FACS using anti-CD3 FITC mAb, anti-CD45 allophycocyanin mAb, anti-CD8 PerCP mAb (all from BD Biosciences), and A2/Mart1 fluorescent soluble tetramers (0.2 μg for 1 h at room temperature; tetramers kindly provided by P. Kourilsky’s laboratory (Pasteur Institute, Paris, France) on a FACSCalibur (BD Biosciences). A2/HIV gag soluble tetramers were used as negative controls of the A2/Mart1-specific staining. LT11 were used as positive controls for the staining with A2/Mart1-soluble tetramers.

The effector function of PBL following in vitro stimulation was evaluated by subjecting the cells to stimulation with T2 or autologous DC pulsed or not with 10 μM of the relevant Mart1/MelanA<sub>26–35</sub> epitope or to the FON tumor line in the presence or absence of MA2.1 mAb neutralizing anti-MART1 antibody. IFN-γ release by PBL was measured in the supernatants of the cultures in IFN-γ ELISA (Immunotech and BD Pharmingen). In the T2 in vitro stimulation assays, PBL function was assessed after sorting, on a FACScsVerage (BD Biosciences). A2/HIV gag soluble tetramers were used as negative controls of the A2/Mart1-specific staining. LT11 were used as positive controls for the staining with A2/Mart1-soluble tetramers.

MelanA/Mart1-specific CD8+ T cell induction in HHD2 mice

Human D<sup>+</sup> (HHD2) mice derived from a strain deficient for mouse β<sub>2</sub>-microglobulin and H-2D<sup>+</sup> molecules and transgenic for a chimeric MHC class I molecule, HLA-A0201/β<sub>2</sub>-microglobulin linked to the human β<sub>2</sub>-microglobulin (24), were provided by F. Lemonnier (Pasteur Institute). Transgenic mice were immunized in the footpad with 50 μl of the vaccine. Different vaccine designs were used (DC-derived exosome (Ex) alone, Ex onto mDC or iDC/peptides). Detailed experimental settings are described in figure legends. Briefly, mouse ExA2+ ‘Mart1 (ExK)’ or mouse ExA2+ ‘Mart1+’ exosomes were either inoculated directly intraderally or pulsed onto 3 ×
Immunocapture assays with an internal standard allowed monitoring of the numbers of MHC class I molecules/exosome batch. Each group of three to five animals was immunized in the footpad with $4 \times 10^8$, $2 \times 10^9$, or $1 \times 10^9$ exosomal MHC class I molecules. Other vaccines consisted of direct injection of peptide-pulsed iDC or mDC HHD2 DC. A single dose of $3 \times 10^5$ DC was loaded with increasing concentrations (from 0.01 to 10 μM) of MelanA/Mart1 26–35 (ELA) peptides. Each experimental group contained three to five mice, and experiments were performed at least three times. Pooled data of the most representative experiments are shown on the graphs.

Mice were immunized once at day 0, and popliteal and inguinal draining LN and contralateral nodes were harvested at day 5. LN mononuclear cells were first stained with A2/Mart1 or A2/HIV gag fluorescent (PE) soluble tetramers (0.2 μg) for 30 min at room temperature in 20 μl of 1× PBS/0.5% BSA (Sigma-Aldrich), then with anti-CD3 FITC mAb and anti-CD8 allophycocyanin mAb (BD PharMingen) for 30 min at room temperature before washing steps and analysis in a FACSCalibur (BD Biosciences). Function of LN-residing T cells following immunization was assessed by challenging LN mononuclear cells to grading doses of Mart1 peptides. Supernatants were collected at 48–72 h to evaluate IFN-γ, IL-4, and IL-10 levels in ELISA (BD PharMingen).

Statistical analyses

Absolute numbers of CD8+ T cells and IFN-γ levels have been analyzed using an ANOVA with Fisher’s exact method. Significances within 95 and 99% confidence interval are depicted on the graphs with * and **, respectively. Comparisons between groups were performed using the nonparametric Kruskal-Wallis test.

Results

Exosomes require DC to activate MHC class I-restricted CTL.

Multivesicular bodies containing exosomes are mostly evidenced in monocyte-derived DC maintained in an immature stage (CD14+, CD1a+, HLA-DRdim, CD80dim, CD86dim, and CD83−) displaying poor allostimulatory capacity in vitro (12) (data not shown). Purification of exosomes contained in culture supernatants was performed using the classical serial ultracentrifugation process or using good manufacturing procedures based on dia- and ultrafiltration steps (23), optimizing yields of exosome harvesting during upscaling. These procedures allowed isolation of a heterogeneous population of vesicles, as assessed in immunoelectronmicroscopy studies (Fig. 1). Exosomes were 60- to 90-nm vesicles expressing high levels of MHC class II molecules (Fig. 1) and tetraspanins such as CD81 (Fig. 1C). For the first time, we show a colocalization of MHC class I with MHC class II molecules on most of the exosomes (Fig. 1B). Constitutive secre-

10⁵ H-2b mDC (DC(Kb)) or mature HHD2 DC (DC(A2)). Immunocapture assays with an internal standard allowed monitoring of the numbers of MHC class I molecules/exosome batch. Each group of three to five animals was immunized in the footpad with $4 \times 10^8$, $2 \times 10^9$, or $1 \times 10^9$ exosomal MHC class I molecules. Other vaccines consisted of direct injection of peptide-pulsed iDC or mDC HHD2 DC. A single dose of $3 \times 10^5$ DC was loaded with increasing concentrations (from 0.01 to 10 μM) of MelanA/Mart1 26–35 (ELA) peptides. Each experimental group contained three to five mice, and experiments were performed at least three times. Pooled data of the most representative experiments are shown on the graphs.

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The yields of exosomal MHC class I molecules recovered in a 24-h DC culture supernatant were evaluated using an immunocapture assay (22) with the anti-HLA-A-B-C mAb. From $2 \times 10^{11}$ to $10^{12}$ MHC class I molecules were recovered from $3 \times 10^{7}$ to $10^{8}$ human iDC culture/24 h.

FIGURE 3. DC pulsed with ExA2/H1/Mart1 induce Mart1-specific CTL expansion in vitro. PBL were weekly stimulated with autologous MD-DC pulsed with either autologous ExA2/H1/Mart1 or allogeneic ExA2/H1/Mart1, or with 1–10 μM Mart126–35 peptides, as described in M&M. Upper panels. Show staining of CD3+/CD8+ T cells with A2/Mart1 fluorescent tetramers at day 21 (A, NV 1), day 14 (B, NV 2), or day 7 (C, NV 3; D, NV 4). Negative controls included irrelevant A2/HIVgag tetramers (data not shown). Lower panels. Show IFN-γ release (A and B) by expanded lymphocytes stimulated with autologous MD-DC ± Mart1 peptide (10 μM) or cytotoxicity assay (C and D) against 51Cr-labeled FON melanoma cells in the presence or absence of neutralizing anti-HLA-A2 molecules (MA2.1 mAb). Depicted values represent the mean of duplicate wells (± SEM). Each panel represents one experiment from an individual NV.
Direct loading of the exosomal MHC class I molecules with synthetic CTL-defined epitopes, i.e., Mart1\(^{26-35}\) (ELA), was undertaken following acid elution of the exosomal pellet and incubation with graduating concentrations of peptides at various pH. Optimal loading of exosomes with Mart1\(^{26-35}\) Peptide (ELA) peptides was obtained for concentrations ranging between 1 and 100 \(\mu\)M, at pH 5.1, as assessed using HLA-A2-binding assays in the presence of a competitive reference epitope (data not shown). To demonstrate the functionality of the HLA-A2/ELA complexes harbored on exosomes, HLA-A2\(^{+}\) or HLA-A2\(^{-}\) exosomes loaded with ELA peptides (ExA2\(^{+}/\)ELA or ExA2\(^{-}/\)ELA, respectively) were incubated with the HLA-A*0201-restricted, ELA-specific CTL clone LT11 (14). When directly loaded onto LT11, ExA2\(^{-}/\)ELA did not trigger IFN-\(\gamma\) production from LT11 at any exosome dose level (Fig. 2). However, when loaded onto immature HLA-A2\(^+\)DC, ExA2\(^{-}/\)ELA induced a significant IFN-\(\gamma\) release by LT11 (Fig. 2). ExA2\(^{-}/\)ELA-mediated T cell activation was peptide dependent because neither ExA2\(^{+}/\)ELA (data not shown) nor unpulsed exosomes (ExA2\(^{+}\)) were efficient (Fig. 2). Moreover, ExA2\(^{-}/\)ELA-mediated LT11 activation was abrogated with an anti-MHC class I Ab (W6.32) (data not shown). To demonstrate that CTL activation results from the transfer of exosomal MHC class I/peptide complexes to DC, we showed that MHC class I molecules of the recipient DC are dispensable for LT11 activation, while exosomal MHC class I molecules are required. Indeed, loading of HLA-A2\(^+\) DC with ExA2\(^{-}/\)ELA (at the highest dose level, i.e., 10\(^{10}\)) did not trigger LT11 IFN-\(\gamma\) production (Fig. 2). Conversely, by loading HLA-A2\(^-\) DC with ExA2\(^{-}/\)ELA at various exosome dosages, we showed that ExA2\(^{-}/\)ELA promoted IFN-\(\gamma\) production by LT11 as efficiently on HLA-A2\(^-\) DC as on HLA-A2\(^+\) DC, as a function of exosomal MHC class I molecules (Fig. 2).

Altogether, these data suggest that exosomes vehicle functional MHC class I/peptide complexes that were transferred from DC to naive DC for MHC class I-restricted T cell activation.

**Exosomal MHC class I complexes prime naive CD8\(^{+}\) T cells in vitro**

We next investigated whether exosomal MHC class I molecules could elicit primary Mart1 (ELA)-specific CD8\(^+\) T cell responses in vitro. PBL were weekly stimulated with T2 pulsed with ExA2\(^+/\)Mart1 or ExA2\(^-/\)Mart1. PBL were stained with A2/Mart1-soluble tetramers at day 14. Negative controls included irrelevant A2/HIV\(gag\) tetramers (data not shown). The A2/Mart1 tetramer-positive and -negative cells were then cell sorted on a FACSVantage and cocultured with T2 cells pulsed or not with 10 \(\mu\)M of Mart1\(^{26-35}\). Twenty-four-hour coculture supernatants were assayed in IFN-\(\gamma\) ELISA. Depicted values represent the mean of duplicate wells (\(\pm\)SEM).

**FIGURE 4.** The TAP-deficient lymphoblastoid T2 line pulsed with ExA2\(^+/\)Mart1 induces Mart1-specific CTL expansion in vitro. PBL were weekly stimulated with T2 pulsed with ExA2\(^+/\)Mart1 or ExA2\(^-/\)Mart1. PBL were stained with A2/Mart1-soluble tetramers at day 14. Negative controls included irrelevant A2/HIV\(gag\) tetramers (data not shown). The A2/Mart1 tetramer-positive and -negative cells were then cell sorted on a FACSVantage and cocultured with T2 cells pulsed or not with 10 \(\mu\)M of Mart1\(^{26-35}\).

CD8 FITC

T2 + ExA2\(^+/\)Mart1

0.3 \%

T2 + ExA2\(^+/\)Mart1

T2

A2/Mart1 PE

T2 + ExA2\(^+/\)Mart1

T2 / Mart1

T2 / Mart1

T2 + Mart1 1.5 \%

T2 / Mart1

IFN-\(\gamma\) (IU/\(\mu\)l)

CD8 FITC

0 10 20 30 40
I of the recipient APC are dispensable, we used the TAP-deficient lymphoblastoid line T2 for the transfer of exosomal MHC class I molecules. Up to 1.5% CD8+ T cells stained with the A2/Mart1-specific tetramers when naive PBL were stimulated using 10^{10} ExA2^{ELA} vs only 0.3% using ExA2^{\text{naive}} (Fig. 4). Following cell sorting of the positive fraction using the specific tetramers, CD8+ T cells produced IFN-γ when challenged with T2 pulsed with Mart1 epitopes, but not with unpulsed T2 cells. We confirmed that the negative fraction of the sorting was not Mart1 specific.

Exosomes harbor functional MHC class I/peptide complexes, but require DC for efficient priming of MHC class I-restricted CTL in vitro.

**Exosomes require DC to prime naive CD8+ T cells in vivo**

To examine MHC class I-restricted presentation by exosomes in vivo, we took advantage of the HHD2 transgenic mouse model, which expresses HLA-A2.1 monochain molecules in H-2c context, therefore exhibiting significant capacity to develop HLA-A2.1-restricted CTL responses (26). In such mice deleted from the endogenous β2-microglobulin and H-2Db mouse genes, the β2-microglobulin is human and covalently linked to the HLA-A2 H chain composed of the human α1 and α2 domain. In contrast, the α3 domain remains murine. CD8+ T cells constitute only 2–5% of the LN cellularity and exhibit a biased T cell repertoire compared with naive LN and unpulsed mDC (HHD2); Fig. 5). Backing CTLs among CD8+/HLA-A2 tetramers was not significant (data not shown). As mentioned for MD-DC human cultures, up to 2 × 10^6 exosomal MHC class I molecules were purified from 10^6 mouse BM-DC cultures in 24 h. Immunogenicity of HHD2-derived exosomes was assessed in HHD2 mice. Analyses of CD3+CD8+ T cell binding to A2/Mart1-soluble fluorescent tetramers were performed in the draining LN, 5 days after inoculation of exosomes in the footpad. Immunization with 10^{10}–10^{11} exosomal A2^{ELA} complexes did not allow specific CTL expansion in LN (Fig. 5). Even in prime-boost settings with two injections of exosomes 10 days apart, no specific CTL expansion was observed (data not shown). In contrast, when loaded onto mDC (LPS-activated HHD2 (DC-A2) or H-2Kb wild-type DC (DC(Kb))), exosomes bearing 10^{9}–10^{10} MHC class I molecules significantly promoted the differentiation of 4–5% specific CTLs among CD8+ T lymphocytes (p < 0.01; Fig. 5). Expansion of specific CD8+ T cells following immunization with DC(Kb)/ExA2^{ELA} was peptide dependent because unpulsed exosomes DC(Kb)/ExA2^{ELA} did not significantly induce specific CTL proliferation (p > 0.05 compared with naive LN and p < 0.01 compared with DC(Kb)/ExA2^{naive}; Fig. 5A). The HLA-A2 molecules harbored by exosomes were indispensable because ExA2 derived from wild-type B6 BM-DC and pulsed with ELA epitopes (ExA2^{ELA}) did not significantly expand tetramer-binding CD8+ T cells (p > 0.05 compared with naive LN and p < 0.05 compared with ExA2^{ELA}; Fig. 5A). In this experimental setting, mDC(HHD2) are also efficient-presentation cells with significant induction of Mart1-specific CTLs after pulsing with 10 μM Mart1_{26–35} epitopes (up to 10% A2/ELA-specific CTL among CD8+ T cells, p < 0.01 compared with naive LN and unpulsed mDC (HHD2); Fig. 5). Background staining using nonspecific A2/gag tetramers was not significant (Fig. 5B). It is noteworthy that exosome transport by DC is efficient across species barriers because loading of mDC(Kb) with human ExA2^{ELA} generated from NV allowed expansion of specific CD8+ T cells in HHD2 mice (36).

Altogether, exosomes bear functional MHC class I/peptide complexes that can be transferred to DC to promote expansion of class I-restricted CTL in vivo.

**Peptide-based vaccine devices for efficient CTL priming in vivo**

Ideal vaccines should combine CTL with helper epitopes, and be molecularly defined, stable, and out-of-shelf reagents. Efficient immunization with CTL-defined epitopes was reported using peptides associated with synthetic (such as CpG sequences) or natural adjuvants (mDC) in mouse. Therefore, we first analyzed HLA-A2.1-restricted, Mart1-specific CTL priming in HHD2 mice following immunization with 3 × 10^6 iDC or mDC propagated from HHD2 bone marrow (iDC(A2), mDC(A2), respectively) and pulsed with 0.01- to 10-μM ranges of Mart1_{26–35} peptides (only shown with 1 μM for iDC). In the absence of LPS activation, iDC(A2) express low surface expression levels of I-A^b class II, CD40, CD80, and CD86 molecules (data not shown). Following 24 h of LPS stimulation, BM-DC acquire an activated phenotype (mDC(A2)) with surface expression of I-A^b and costimulatory molecules associated with allostimulatory capacities in vitro (data not shown). Both mDC(A2) and iDC(A2) elicited significant Mart1-specific CTL expansion in HHD2 mice (Fig. 6A). In both cases, the peptide concentration threshold necessary to initiate CTL expansion appeared to be above 0.01 μM (Fig. 6A). Importantly, only mDC (HHD2) and not iDC (HHD2) promoted Tc1 IFN-γ-producing CTL (Fig. 6B). The threshold for mDC-mediated Tc1 differentiation was above 0.1 μM peptide concentrations (p > 0.05 between 0.1 μM mDC and unloaded mDC, p < 0.01 between 1 μM mDC and unloaded mDC, p < 0.01 between 10 μM mDC and unloaded mDC; Fig. 6B) with a plateau reached at 1 μM of Mart1_{26–35} (p > 0.05 between 1 and 10 μM mDC). IFN-γ production of LN-residing CTL was peptide dependent because a Mart1 peptide dose response for IFN-γ secretion was demonstrated (data not shown).

Because we demonstrated that efficient MHC class I-restricted Ag presentation could be achieved using mDC and exosomes, we aimed at comparing efficacy of Tc1 differentiation using exosomes vs mDC.

Immunization with exosomes pulsed with Mart1_{26–35} epitopes was performed between 10^8 and 10^{10} MHC class I molecules in the presence of mDC (HHD2) (Fig. 6). As already reported, Fig. 5 using H-2b allogeneic mDC, exosomes loaded onto syngeneic mDC(A2) significantly expanded ELA-specific CTL (Fig. 6A, p < 0.01) that differentiate into potent Tc1 (Fig. 6B, p < 0.01) with a plateau reached at 2 × 10^9 MHC class I molecules (p > 0.05 between 2 × 10^8 ExA2^{ELA} and 1 × 10^{10} ExA2^{ELA}; Fig. 6). Tc1 differentiation was peptide dependent because unpulsed exosomes (ExA2^{naive}) did not significantly promote IFN-γ-secreting CD8+ T cells (Fig. 6B). Once again, MHC class I/peptide complexes from exosomes were indispensable because ExA2^{ELA} pulsed onto mDC (HHD2) did not significantly induce differentiation of specific Tc1 (p > 0.05 compared with ExA2^{naive} and p < 0.05 compared with ExA2^{ELA}; Fig. 6B). In all cases, IFN-γ secretion by LN-residing CD8+ T cells was peptide dependent (data not shown).

Finally, immunogenicity of 2 × 10^9–10^{10} exosomal ExA2^{ELA} complexes was compared with that mediated by 3 × 10^5 mDC(A2) pulsed with saturating concentrations of peptides in HHD2 mice. Indeed, in our model system, a plateau of Tc1 differentiation was achieved with mDC using ~0.3–0.5 × 10^7footpad injection (data not shown). The threshold for exosome efficacy in Tc1 priming was ~2 × 10^9 MHC class I molecules with no additional benefit at 10^{10} MHC class I complexes when pulsed onto mDC(A2). Importantly, there is no statistically significant
FIGURE 5. Exosomes require DC to induce expansion of specific CD8⁺ T cells in vivo. HHD2 mice were immunized at day 0 in the footpad with autologous (EXA₂⁺) or allogeneic exosomes (EXK⁺) pulsed with Mart₁₂₆–₃₅ peptides (ELA) or not pulsed (0) at a dosage of 10¹⁰ MHC class I molecules (unless otherwise specified). Alternatively, exosomes were loaded onto allogeneic H-2b mature BM-DC. Positive controls were mice inoculated with autologous mature BM-DC pulsed with 10 μM of Mart1 (ELA). −, Represents naive LN. At day 5, mononuclear cells (Figure legend continues)
difference between: 1) $2 \times 10^6$ or $10^{10}$ ExA2$^{+/\text{ELA}}$ and mDC pulsed with 0.1–10 $\mu M$ Mart1 regarding the expansion of A2$^+$Mart1$^+$ tetramer-binding CD8$^+$ T cells ($p > 0.05$; Fig. 6A), and 2) $2 \times 10^6$ or $10^{10}$ ExA2$^{+/\text{ELA}}$ and mDC pulsed with 1–10 $\mu M$ Mart1 regarding the priming of Mart1-specific Tc1 ($p > 0.05$; Fig. 6B).

Exosomes can be considered as immunogenic vehicles of MHC class I/peptide complexes secreted by DC for the differentiation of Tc1 cells. Moreover, exosomes mimic the capacity of mDC to initiate synthetic peptide-specific CD8$^+$ T cell responses in vivo.

**Discussion**

Following our study showing that DC-derived exosomes promote T cell-dependent antitumor immune responses in vivo (12), we aimed at elucidating the mechanisms of CTL priming by exosomes in vivo. Our data demonstrate that: 1) exosomal MHC class I/peptide complexes efficiently induce peptide-specific, MHC class I-restricted CD8$^+$ T cell expansion and differentiation in vitro and in vivo; 2) exosome immunogenicity requires transfer onto APCs; 3) CTL priming against a synthetic class I-restricted epitope is achieved as efficiently with mDC- as with DC-derived exosomes.

Exosome production is an elective feature of DC at an immature stage when multivesicular bodies still form in the cytosol. This stage might correspond to macrophagocytosis-mediated microenvironmental scanning and uptake of surrounding Ag for sequestration in MHC class II compartments. After activation, stored Ags are processed and loaded onto neosynthesized MHC molecules (2).

We bypassed the need for endogenous Ag processing by directly pulsing the candidate model Ag onto exosomes. Our ex vivo process of peptide loading onto exosomal HLA-A2.1 molecules was efficient and allowed triggering of peptide-specific CTL clones and priming of naive Mart1-specific, HLA-A2-restricted CD8$^+$ T cells in lymphocytes of several NV. CTL lines differentiated using DC-derived exosomes recognize not only the synthetic peptide, but also the naturally processed Mart1 epitope presented on tumor cells. CTL priming by DC-derived exosomes is efficient and requires activated or mature DC. Indeed, triggering of CTL clones using exosomes required iDC presumably because clones do not need costimulatory signals. Otherwise, LPS-activated human monocyte-derived DC or BM-DC were used for efficient CTL priming in vitro and in vivo. Other reports showed transfer of functional MHC class I/peptide complexes by exosomes to DC (28, 29).

Nevertheless, while MHC class I molecules of the recipient DC are dispensable for efficient exosome-mediated CTL priming, functional exosome-associated MHC class I molecules are needed in vitro and in vivo. The evidence that exosomes are even more efficient at CTL priming than micromolar ranges of peptides in CpG oligomers further supports that: 1) exosome bioactivity cannot be accounted for by free HLA-A2-unbound peptides; 2) exosomes require innate immune responses eventually leading to DC activation (36).

Adjuvantization of exosomes with mDC allowed recruitment (2–5×; data not shown) of CD4$^+$ T cells in the draining LN, although no defined MHC class II epitopes were copulsed onto exosomes in our experiments. Nevertheless, such a CD4$^+$ T cell help might be required for the Mart1-specific CTL priming that we observed because exosomes injected in the absence of adjuvants (i.e., mDC or CpG) did not allow expansion of CD4$^+$ T cells in popliteal homolateral nodes. Importantly, adjuvantization of exosomes with CpG was absolutely required for exosomes to mediate tumor rejection (36).

Membrane transfer has been abundantly reported in vitro, in systems requiring or not cell to cell contacts (16). The observation that alive DCs transfer antigenic material to other DCs has already been suggested. Harshyne et al. (5) have shown that genetically modified monkey DCs transfer gp100 to human DCs for cross-presentation. In this study, antigenic transfer was mediated by plasma membrane material and inhibited by a 0.4-$\mu$m-diameter filter. Knight et al. (6) have shown that DC acquire Ag from cell-free DC supernatant. Our data underscore that exosomes could be mediators of antigenic exchange in between DC and APC. The in vivo relevance of this hypothesis remains unclear. It is conceivable that following Ag uptake by DC in the periphery, DC migration be hampered and processed Ags be released via exosomes for appropriate Ag presentation in LN. It has been shown in mice that CD8$^+$ lymphoid DC could generate an immune response after s.c. injection without homing to draining LN (30). Likewise, when antigenic dose is limiting (i.e., low viral load), it is conceivable that a system of antigenic spreading or amplification might exist.

In these pathophysiological conditions, one might expect that processing of the exogenous Ag by a few DC would be sufficient to overload the surrounding DC with functional MHC class I and II/peptide complexes for initiation of T cell responses. Such a process would require an efficient secretion of preformed immunological synapses that should be uptaken by other APC in a targeted and saturable manner. At first, we showed that: 1) an iDC can secrete in vitro at least as many MHC class I molecules as what is presented on its plasma membrane (up to $10^4$ MHC class I molecules/24 h in vitro); 2) such exosomal MHC class I molecules are in a bioactive conformational form. We set up our in vivo priming system showing that up to $3 \times 10^9$ mDC pulsed with saturating amounts of synthetic peptides (1 $\mu M$) reach a plateau for Tc1 differentiation. Exosome-mediated Tc1 priming reached a plateau at $-2 \times 10^9$ MHC class I/peptide complexes. In this model system, $-2 \times 10^9$ exosomal MHC class I molecules released by $10^5$ DC are as efficient as $3 \times 10^5$ mDC loaded with saturating $\mu M$ ranges of peptides for the expansion and differentiation of synthetic peptide-specific Tc1 lymphocytes in vivo. Western blot analyses of proteins contained in exosomes and DC lysates using anti-MHC class I Ab appear to indicate that $3 \times 10^5$ MHC class I molecules released in comparison, CD and exosome immunogenicity. However, our data support that exosomal MHC class I complexes elicit potent primary CD8$^+$ T cell responses with higher affinity for the TCR than peptides.

Second, unloaded exosomes can compete with peptide-loaded exosomes for T cell activation in vitro (our unpublished data), suggesting that exosome uptake by DC might be saturable and
FIGURE 6. Legend continues
receptor mediated. Third, the $\alpha_\text{L}\beta_2$ and $\alpha_\text{M}\beta_2$ integrin-binding lactivin has been shown to be a relevant candidate molecule for the uptake and/or clearance of apoptotic bodies (33) and is over-expressed on mouse DC-derived exosomes (18). However, the role of lactadherin for exosome targeting to DC remains to be elucidated. Interestingly, Denzer et al. (34) were able to show that follicular DC (FDC) that do not synthesize MHC class II molecules can nevertheless bear MHC class II molecules at their surface in vivo. They provide evidence for elective binding of B lymphocyte-derived exosomes to FDC, but not to other APC in vitro, eluding to the targeted transfer of exosomal MHC class II molecules from B cells to FDC.

We have recent evidence of the presence of exosomes in vivo in peritonal or pleural effusions of cancer patients (22). Such exosomes harvested from biological fluids bear high levels of candidate tumor Ags, MHC class I, and heat shock protein 70–90 molecules. Ascitis-derived exosomes could elicit MHC class I-restricted, tumor-specific T cell recognition in autologous settings in vitro. In this setting, MHC class I-restricted cross-presentation of tumor Ags by autologous DC and direct presentation of preformed MHC class I/peptide complexes harbored on exosomes could account for the immunogenicity of tumor ascitis-derived exosomes.

Regardless of their immunological relevance, exosomes represent valuable vectors of MHC class I or II molecules (35) that can be loaded with synthetic peptides of choice. Long-term allogeneic DC culture systems or cell lines are awaited to generate large amounts of out-of-shelf exosomal reagents exhibiting MHC complexes of given relevance in various immunization protocols.

Acknowledgments

We thank Dee Wei Hsu for providing some materials and methods for exosomal GMP production, and C. Thiéry for critical review of the manuscript and fruitful discussions. We are indebted to Dominique Gambaud, Annick Duval, and Christian Loy from Etablissement Français du Sang Nord de France for assistance with the apheresis, and to P. Ardouin and its animal facility for mice handling.

References


