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Dendritic Cell–Derived Exosomes Need To Activate Both T and B Cells To Induce Antitumor Immunity

Tanja I. Näslund, Ulf Gehrmann, Khaleda R. Qazi, Mikael C. I. Karlsson, and Susanne Gabrielsson

Exosomes are secreted membrane nanovesicles of endosomal origin and are considered potential cancer vaccine vectors. Phase I clinical trials have been successfully conducted with tumor peptide–loaded exosomes derived from dendritic cells (dexosomes), and a phase II clinical trial is ongoing. However, much is still unknown regarding the in vivo role of dexosomes and whether their immunogenicity can be enhanced. We previously reported that dexosomes induce CD4+ T cell responses in a B cell–dependent manner, suggesting that immunization with dexosomes carrying only T cell peptides induce suboptimal immune responses. In this study, we show that CD8+ T cell responses were induced in vivo when mice were immunized with protein-loaded, but not peptide-loaded, dexosomes. We also show that the cytotoxic T cell response was totally dependent on CD4+ T cells and, interestingly, also on B cells. Mice deficient in complement activation and Ag shuttling by B cells have lower responses to protein-loaded dexosomes, showing involvement of these B cell–mediated mechanisms. Finally, protein-loaded dexosomes were superior in protecting against tumor growth. In conclusion, proper activation of CD4+ T and B cells needs to be considered when designing cancer vaccines to ensure full potential of the treatment. The Journal of Immunology, 2013, 190: 2712–2719.

Cancer immunotherapy has long been an intense field of investigation. Different approaches have been tested in clinical trials using Abs, cytokines, peptides, viral vectors, tumor cell–based vaccines, and infusion of T cells or dendritic cells (DCs) (http://www.nci.nih.gov and http://www.wiley.co.uk/genmed/clinical). DC-based immunotherapy is an attractive therapy approach because mature DCs act as professional APCs capable of inducing strong T and B cell responses in vivo (1). However, in vitro–generated DCs can be influenced by immune-inhibiting factors released by the tumor environment, leading to deletion or suppression and failure to induce the desired immune response. One way to avoid this is to use DC-derived exosomes (dexosomes), which are inert vesicles that cannot be influenced by the surrounding milieu. They might be more advantageous than DCs as therapy candidates, or they could be used as a complement to DC immunotherapy (2). In addition, it was reported that dexosomes have a longer half-life in vivo after injection than do DCs (3).

Exosomes are ∼100-nm membrane vesicles originating from the endosomal compartment that are released into the extracellular space (4). The interest in exosomes as vaccine vehicles was raised in the 1990s by the discovery that exosomes generated from B cells and DCs were capable of activating CD4+ T and CD8+ T cells (2, 5). Since then, T cells, macrophages (MΦs), mast cells, epithelial cells, platelets, and tumor cells were shown to release exosomes (6). In humans, exosomes have been found in multiple body fluids (7–10), including plasma (11). Two phase I clinical trials with patients with non-small cell lung cancer and melanoma were successfully performed with dexosomes (12, 13), and other trials are under way (14). However, much is still unknown regarding the endogenous role and function of dexosomes and how they activate the immune system in vivo.

Dexosomes can be loaded with proteins or peptides either indirectly, by addition of proteins or peptides to DCs that process the Ag onto the dexosomes, or directly to preformed dexosomes. In phase I clinical trials, dexosomes were loaded with tumor-specific MHC class I and II peptides, both directly and indirectly (12, 13). However, we showed that protein-loaded dexosomes were superior to peptide-loaded ones in activating CD4+ T cells in vivo, an effect due to B cell–mediated CD4+ T cell activation (15). Recent reports showed that an adaptive immune response needs Ag presentation first by DCs and subsequently by B cells before CD4+ T cells can differentiate into T follicular helper cells (16). Thus, immunizing with dexosomes containing only T cell–specific peptides, devoid of B cell epitopes, will induce suboptimal immune responses with no potent germinal center formation. In a cancer vaccine setting, efficient CD8+ T cell activation is crucial, and it is still not known to what extent CD8+ T cells are dependent on Th cells or B cells after dexosome immunization. Thus, determining how dexosomes induce a potent cytotoxic response in vivo is important for the design of future dexosome-based cancer vaccines.

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T.I.N., U.G., S.G., and M.C.I.K designed the study and analyzed the results. T.I.N. and U.G. designed and performed most experiments. K.R.Q performed some experiments. T.I.N. wrote the manuscript, and S.G., U.G., and M.C.I.K revised the manuscript.

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Abbreviations used in this article: BMDC, bone marrow–derived dendritic cell; DC, dendritic cell; Exo-BSA, dexosome generated from BSA-pulsed BMDCs; Exo-OVA, dexosome obtained from OVA protein-pulsed BMDCs; Exo-SIIN, dexosome obtained from SIINFEKL-pulsed BMDCs; FDC, follicular dendritic cell; KI, Karolinska Institutet; MΦ, macrophage; μMT, B cell knockout mice; MZB, marginal zone B cell; RT, room temperature.

The Journal of Immunology
In this study, we show that dexosomes induce CD8+ T cell responses in a CD4+ T cell–dependent manner. Importantly, B cells were also shown to be necessary for the optimal activation of CD8+ T cells. Thus, protein-loaded dexosomes, containing both Th and B cell epitopes, were superior to MHC class I peptide–loaded dexosomes in both the induction and activation of specific cytotoxic T cell responses in vivo. Moreover, protein-loaded dexosomes protected against tumor growth to a larger extent than did peptide-loaded ones. We propose that an effective dexosome-induced cytotoxic immune response is dependent on CD4+ T cell activation mediated by B cells. The consequence of this is that B cell activation to cancer-derived Ags has to be taken into account in cancer vaccine regimens.

Materials and Methods

Mice and immunizations

CD4 knockout (CD4−/−) mice, OT-I/Rag2−/− mice, C57BL/6 Ly5.1 (CD45.1+) mice, corresponding wild type mice (C57BL/6), CD19−/− mice on Sv129 background (kindly provided by Nils Lycke, University of Gothenburg, Sweden), and subline wild type mice Sv129 were bred and kept at the animal facility at Karolinska Institutet (KI). B cell knockout mice (μMT) (C57BL/6 background) were bought from The Jackson Laboratory (Bar Harbor, ME). The mice were immunized i.v. with 40 μg dexosomes under pathogen-free conditions in a total volume of 100 μl PBS. Animal care and treatment were in accordance with standards approved by the local ethics committee.

Generation of dexosomes

Bone marrow–derived DCs (BMDCs) were generated from C57BL/6 mice by culturing bone marrow cells at 37°C, 5% CO2 at 4 h and kept at the animal facility at Karolinska Institutet (KI). B cell knockout mice (μMT) (C57BL/6 background) were bought from The Jackson Laboratory (Bar Harbor, ME). The mice were immunized i.v. with 40 μg dexosomes under pathogen-free conditions in a total volume of 100 μl sterile PBS. Animal care and treatment were in accordance with standards approved by the local ethics committee.

Sucrose gradient

A total of 300 μg dexosomes was added on top of a linear sucrose gradient (0.25 M sucrose/20 mM HEPES [pH 7] and 2 M sucrose/20 mM HEPES [pH 7]; Sigma–Aldrich) and centrifuged for 20 h at 80,000 × g (Beckman Coulter) at 4°C. One-milliliter fractions were collected, and the fraction density was determined by refraction index measurements. A total of 200 μl of each fraction was added to uncoated latex beads, stained with CD81 Abs, and analyzed by flow cytometry, as described above.

Nanosight

The size of the dexosomes was measured using an LM10HSB system from NanoSight (Amesbury, UK), equipped with a 405-nm laser running an NTa 2.2 analytical software package. The dexosome preparations were diluted serially in PBS, and the software settings were set to minimum expected size 50 nm, multiple detection threshold, and automatic minimum track length. A total of 421–2177 completed tracks was recorded in the different dilutions before analyses.

In vitro proliferation

Single-cell suspensions were prepared from spleens from OT-I mice and treated with ACK buffer (0.8% NH4Cl, 0.1% KHC03, 0.1 mM EDTA [pH 7.3]) to lyse RBCs and then were washed and resuspended in PBS. The splenocytes were labeled with 6 μM CFSE in PBS for 10 min at 37°C and then washed and resuspended in RPMI 1640 medium supplemented with 200 mM L-glutamine, 100 IU/ml penicillin-streptomycin (Thermo Scientific), and 10% FCS (HyClone) (culture medium), and 2 × 10^5 splenocytes were added per well using a 48-well plate. Dexosomes were diluted in culture medium and added to the OT-I splenocytes in duplicates at a final concentration of 2.5, 10, or 50 μg/ml before incubation at 37°C, 5% CO2 for 5 d. On day 3, Con A (Sigma–Aldrich) was added as a positive control in 2 μl/ml. On day 5, the cells were stained with CD8–PerCPCy5.5 and CD3–allophycocyanin Abs, and proliferation of the CD8+CD3+ cells was analyzed by flow cytometry.

In vitro cytotoxic T cell lysis assay

Freshly isolated splenocytes were stimulated in vitro with 10 μM SIINFEKL peptide (Innovagen) in culture medium for 7 d at 37°C, 5% CO2. Thereafter, the lymphocytes were added to V-shaped 96-well plates and diluted 3-fold. EL-4 cells were loaded with 10 μM SIINFEKL peptide at 37°C for 1 h before washing and labeling with 0.3 μM CFSE, as described above. Unpulsed EL-4 cells were labeled with 6 μM CFSE. Unlabeled EL-4 cells (1000 cells) and SIINFEKL–pulsed EL-4 cells (1000 cells) in culture medium were added per well to the 96-well plate. The recovery of CFSE–labeled SIINFEKL–loaded EL-4 cells was measured after a 4-h incubation (37°C, 5% CO2) using flow cytometry. Specific killing (%) was determined by (1 − %CFSE peptide/%CFSE no peptide) × 100.

In vivo cytotoxic T cell lysis assay

C57BL/6 mice (CD45.2+) were immunized i.v. with 40 μg dexosomes. At day 6 postdexoimmunization, splenocytes from C57BL/6 Ly5.1 (CD45.1+) mice were labeled with CFSE, with or without SIINFEKL peptide, at different concentrations: 2 μg SIINFEKL/0.3 μM CFSE or no peptide/6 μM CFSE. Thereafter, the splenocytes were injected i.v. at a 1:1 ratio (total of 10^7 cells). The following day, spleens were collected, and 10^7 splenocytes were stained with CD45.1-PE Ab. A total of 10^5 CD45.1–positive cells was collected and analyzed using flow cytometry and FlowJo software. Specific killing (%) was determined by (1 − %CFSE peptide/%CFSE no peptide) × 100.

Pentamer staining

A total of 3 × 10^6 freshly isolated splenocytes was stained with 0.1 μM PE-labeled H-2Kd/SIINFEKL pentamer (ProImmune, Oxford, U.K.) and CD16/32 Fc Block (BD Biosciences). The cells were washed and stained with CD8ε–PerCP and CD19–FITC Abs (BD Biosciences) and then washed and resuspended in PBS. A total of 10^6 CD8+ FITC-negative cells was collected for enumeration of H-2Kd/SIINFEKL pentamer–positive cells by flow cytometry.

ELISPOT analysis

Splenocyte single-cell suspensions were treated with ACK lysing buffer and resuspended in culture medium. Splenocytes (2 × 10^6) from individual mice were added to Multiscreen IP plates (Millipore, Billerica, MA) coated with anti-mouse IFN-γ Ab (AN18; Mattech, Nacka Strand, Sweden), according to the manufacturer’s instructions, and stimulated with medium,
2 μg/ml SIINFEKL peptide (Innovagen), or 2 μg/ml Con A (Sigma-Aldrich) for 22 h. The plates were developed with biotinylated anti-mouse IFN-γ Ab (R4-6A2), streptavidin-ALP, and BCIP-Plus (Mabtech). The spots were counted using an EliSpot reader (AID Diagnostika, Straßburg, Germany) and expressed as spot forming cells/10^6 splenocytes, 10^6 CD8^+ T cells, or 10^6 lymphocytes.

In vivo proliferation

Single-cell suspensions were generated from spleens of OT-I mice (CD45.1^+^) and labeled with 6 μM CFSE. A total of 1 × 10^6 CFSE-labeled OT-I splenocytes was injected i.v. in a total volume of 100 μl in C57BL/6 mice (CD45.2^+^). The following day, 40 μg dexosomes was injected i.v. The spleens were collected 5 d postdexosome immunization, splenocytes were stained with CD45.1 Ab, and 10^6 CD45.1-positive cells were collected and analyzed by flow cytometry.

Alternatively, wild type and CD4^+/-^- mice were immunized with 40 μg dexosomes i.v. and given BrdU in their drinking water from day 0 to day 1, day 1 to 3, day 3 to 5, or day 5 to 7 before sacrifice at day 1, 3, 5, or 7. Freshly isolated splenocytes were stained with a BrdU staining kit (BD Biosciences), according to the manufacturer’s instructions, as well as PE-labeled H-2K^b/-SIINFEKL pentamer (ProImmune) and CD16/32 Fc Block (BD Biosciences). The cells were washed and stained with CD8^-allophycocyanin/Cy7 and B220-PerCP/Cy5.5 (BD Biosciences) and analyzed with flow cytometry. A total of 10^6 splenocytes was collected to determine the number of BrdU and H-2K^b/-SIINFEKL–pentamer positive cells. IFN-γ ELISPOT analysis was also performed, as described above.

PKH67 labeling of dexosomes

Dexosomes were pelleted by ultracentrifugation and resuspended in Diluent C before addition of PKH67 lipid dye. To control for unspecific labeling by PKH67, the same amount of lipid dye was solubilized in Diluent C. The reaction was stopped by adding 1% BSA before ultracentrifugation. A total of 1 × 10^6 splenocytes from C57BL/6 mice was cocultured with 20 μg PKH67-labeled dexosomes or PKH67-labeling control for 4 h at 37°C, 5% CO_2 before staining for marginal zone B cells (MZB) (B220^-, CD21^+, CD23^-), follicular B cells (B220^+, CD21^-, CD23^+), MØs (B220^-/CD3^-, F4/80^-), DCs (B220^-/CD3^-, MHCII^+, CD11c^-), and T cells (TCR^b^-/CD3^-) and flow cytometry analysis.

B16/OVA melanoma tumor model

Mice were immunized i.v. with 40 μg dexosomes either with a single injection (day 14) or repeated injections (days 0 and 14). On day 21, 1.5 × 10^5 B16/OVA melanoma cells, a kind gift from Dr. Edith Lord and NY), were injected s.c. in the flank. Tumor growth was monitored regularly, and mice were euthanized when tumors reached 1000 mm^3 in size. Tumor and spleen were removed, and single-cell suspensions were stained with a BrdU staining kit (BD Biosciences), according to the manufacturer’s instructions, as well as PE-labeled H-2K^b/-SIINFEKL pentamer (ProImmune) and CD16/32 Fc Block (BD Biosciences). The cells were washed and stained with CD8^-PerCP and B220-allophycocyanin Abs (BD Biosciences) and analyzed by flow cytometry. IFN-γ ELISPOT analysis was performed, as described above.

Statistics

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). To test for statistical significance, the nonparametric two-tailed Mann–Whitney analysis was performed, with the exception of Fig. 6B, for which the Kruskal–Wallis and Dunn multiple-comparison tests were used.

Results

Phenotype of bone marrow–derived OVA- and SIINFEKL-loaded dexosomes

Dexosomes were obtained from OVA protein (Exo-OVA) or CD8^+ T cell–specific OVA peptide (SIINFEKL; Exo-SIIN) pulsed, LPS stimulated, BMDCs from wild type mice. Dexosomes were coated on anti-CD9^-coated latex beads and evaluated for the presence of typical exosome markers, such as CD9 and CD81, as well as immune-stimulatory markers, such as MHC class II, CD54, CD80, CD86, and SIINFEKL-loaded H-2K^b complex, by Ab staining and flow cytometry. Tetraspanins (CD9 and CD81), as well as immune-stimulatory markers, could be detected in all DC (data not shown) and dexosome preparations (Fig. 1A). Moreover, the markers were detected at similar levels with no statistical difference, irrespective of OVA or SIINFEKL loading. To confirm the density of the dexosomes, they were layered on sucrose gradients, and the sucrose fractions were added to uncoated latex beads, stained for CD81, and analyzed by flow cytometry. The dexosomes were found only in fractions corresponding to typical exosome densities (1.11–1.19 g/ml) (5) (Fig. 1B). Similar data were also generated by CD80 and MHC class II staining (data not shown). The size of the dexosomes was verified by NanoSight measurements, and vesicles with a mean size of 130 nm were detected in both dexosome preparations (Fig. 1C). Thus, Exo-OVA and Exo-SIIN were similar in density and size and displayed typical surface markers at similar levels. In addition, dexosomes generated from BSA protein–pulsed BMDCs (Exo-BSA) displayed similar phenotypic markers, density, and size as Exo-OVA and Exo-SIIN (data not shown).

Both OVA- and SIINFEKL-loaded dexosomes induce SIINFEKL-specific CD8^+ T cell proliferation in vitro

To investigate the potential of Exo-OVA and Exo-SIIN to induce CD8^+ T cell proliferation in vitro, splenocytes from OT-I mice, transgenic for SIINFEKL-specific CD8^+ T cells, were labeled with CFSE and cocultured with dexosomes at different concentrations (2.5, 10, or 50 μg/ml). Strong OT-I cell proliferation was detected in the presence of Exo-OVA or Exo-SIIN, but not of Exo-BSA, with a similar magnitude of total proliferation induced by both types of dexosomes (Fig. 2A). However, although Exo-OVA and Exo-SIIN induced similar bulk proliferation of OT-I cells, the capacity to induce multiple rounds of cell division differed. Exo-OVA induced more than four cell divisions in the majority of responding cells, whereas a more heterogeneous proliferation pattern was detected after Exo-SIIN stimulation (Fig. 2B).
Loading of DCs with 10-fold more SIINFEKL peptide did not induce dexosomes that differed in the capacity to induce proliferation of OT-I cells (data not shown). In conclusion, OVA- and SIINFEKL-loaded dexosomes, but not BSA-loaded dexosomes, induced proliferation of SIINFEKL-specific CD8+ T cells in vitro. However, dexosomes loaded with whole-OVA protein induced more rounds of cell division than did peptide-loaded ones.

OVA-loaded, but not SIINFEKL-loaded, dexosomes activate CD8+ T cell responses in vivo

We reported previously that OVA-specific CD4+ T cells expand in vivo after Exo-OVA immunization but not when dexosomes loaded with the CD4+ T cell OVA epitope (OVA253–339) were used (15). To test the capacity of SIINFEKL-specific CD8+ T cells to proliferate in vivo after exosome immunization, CFSE-labeled OT-I cells were injected into wild type mice that were immunized with Exo-OVA, Exo-SIIN, or Exo-BSA the following day. The proliferation of OT-I cells was analyzed in spleen by flow cytometry 5 d after exosome immunization (Fig. 3A). Interestingly, no proliferation of OT-I cells was detected in mice immunized with Exo-BSA or Exo-SIIN. In contrast, mice immunized with Exo-OVA displayed strong OT-I proliferation, which was significantly higher than in mice immunized with Exo-SIIN ($p < 0.0001$).

Together, these data and our findings in the previous study (15) show that adoptively transferred OVA-specific CD8+ and CD4+ T cells only proliferate in vivo after injection of dexosomes containing whole-OVA Ag. To evaluate the efficiency of dexosomes to stimulate endogenous CD8+ T cell responses in vivo, wild type mice were immunized with Exo-OVA, Exo-SIIN, or Exo-BSA. CD8+ T cell responses were measured in spleen 7 d postimmunization by H-2Kb/SIINFEKL pentamer staining (Fig. 3B), IFN-γ ELISPOT (Fig. 3C), and in vitro CTL assay (Fig. 3D). Neither Exo-BSA nor Exo-SIIN immunization induced any H-2Kb/SIINFEKL pentamer–positive CD8+ T cells or SIINFEKL-specific IFN-γ-producing cells. In contrast, Exo-OVA immunization generated significantly higher numbers of pentamer–positive CD8+ T cells ($p < 0.0001$), as well as IFN-γ–producing cells ($p < 0.01$), than did the other groups tested. In line with the results

**FIGURE 2.** Peptide- or protein-loaded dexosomes are both potent stimulators of CD8+ T cell proliferation in vitro. The capacity of Exo-OVA, Exo-SIIN, or Exo-BSA to induce SIINFEKL-specific CD8+ T cell proliferation was tested. (A) CFSE-labeled, SIINFEKL-specific OT-I splenocytes were stimulated with increasing doses of dexosomes (2.5, 10, or 50 μg/ml) or Con A for 5 d. Proliferated CD8+CD3+ splenocytes were analyzed for CFSE dilution using flow cytometry. Stimulations were performed in duplicates, and data are pooled from three independent experiments and show mean ± SEM. (B) Protein-loaded dexosomes induce more rounds of proliferation than do peptide-loaded dexosomes. Graphs show individual CFSE-dilution peaks. Data are representative of one of three experiments.

**FIGURE 3.** Only dexosomes loaded with whole OVA protein (Exo-OVA) induce potent CD8+ T cell activation in vivo. (A) Wild type mice were injected with CFSE-labeled OT-I cells and immunized i.v. the following day with 40 μg Exo-OVA, Exo-SIIN, or Exo-BSA. Five days after dexosome immunization the spleens were taken and analyzed for the percentage of proliferated OT-I cells by flow cytometry. (B–D) Wild type mice were immunized i.v. with 40 μg Exo-OVA, Exo-SIIN, or Exo-BSA. (B) Seven days postimmunization, the spleens were taken and analyzed for the presence of SIINFEKL-specific CD8+ T cells by H-2Kb/SIINFEKL–pentamer staining. (C) Splenocytes were stimulated with SIINFEKL peptide for 22 h in an IFN-γ ELISPOT to quantify SIINFEKL-specific IFN-γ-producing cells. Dots represent single mice; Data in (A) are pooled from two independent experiments with two to seven mice/group; data in (B) and (C) are pooled from three independent experiments with 5–10 mice/group. To test for statistical significance, a nonparametric two-tailed Mann–Whitney analysis was performed. (D) In vitro–restimulated splenocytes were incubated for 4 h with unloaded (CFSEhigh) or SIINFEKL–loaded (CFSElow) EL4 target cells at different E:T ratios in an in vitro CTL assay. Specific killing was analyzed by flow cytometry, and the percentage of specific lysis was determined as (1 – %CFSEpeptide/%CFSEno peptide) × 100. Connected dots represent single mice, and data are representative of one of two independent experiments.
generated by pentamer staining and ELISPOT assay, only cells from mice immunized with Exo-OVA could lyse SIINFEKL-pulsed target cells (Fig. 3D). In conclusion, only exosomes loaded with whole-OVA protein could induce a CD8+ T cell response in vivo, indicating that epitopes other than SIINFEKL are needed to generate potent cytotoxic T cell responses.

CD4+ T cells are crucial for dexosome-induced CD8+ T cell responses

Because it was reported that CD4+ T cells are crucial for at least the secondary (17) expansion of CD8+ T cells, we next immunized wild type mice and mice lacking CD4+ T cells (CD4−/− mice) with Exo-OVA or Exo-SIIN and measured the CD8+ T cell response in the spleen after 7 d. Similar to wild type mice, CD4−/− mice were not capable of inducing SIINFEKL-specific CD8+ T cells post–Exo-SIIN immunization, as detected by H-2Kb/SIINFEKL pentamer staining (Fig. 4A), IFN-γ ELISPOT (Fig. 4B), and in vivo CTL assay (Fig. 4C). However, in contrast to wild type mice, no SIINFEKL-specific CD8+ T cell responses were seen in CD4−/− mice after Exo-OVA immunization (Fig. 4A–C). These data show that the generation of CD8+ T cells after dexosome immunization is totally dependent on CD4+ T cells. In addition, regulation of the CD8+ T cell response by CD4+ T cells occurred very early during the immune response (days 3–5), as detected by H-2Kb/SIINFEKL pentamer staining (Fig. 4D) and IFN-γ ELISPOT (Fig. 4E).

B cell activation is important for dexosome-induced CD8+ T cell responses

We showed previously that B cells are important for the activation of CD4+ T cells after dexosome immunization (15). To explore whether B cells are equally important for the generation of CD8+ T cell responses, μMT and wild type mice were immunized with Exo-OVA or Exo-SIIN. Seven days after immunization, the CD8+ T cell response was measured in the spleen. Interestingly, Exo-OVA immunization induced significantly higher numbers of SIINFEKL-specific CD8+ T cells (p < 0.0001) (Fig. 5A) and IFN-γ–producing cells (p < 0.01) (Fig. 5B) in wild type mice in comparison with mice lacking B cells. Thus, these data indicate that B cells are also important for the activation of CD8+ T cells after dexosome immunization but to a lesser extent than are CD4+ T cells (Fig. 4).

B cells have a number of functions, including the capacity to present Ag to T cells, differentiating them into T follicular helper cells, as well as shuttling of Ags (18). Splenic MZBs are specialized in Ag transport and were shown to catch Ag and transport them to the follicular DCs (FDCs) in a complement-dependent manner (19). To investigate what types of cells take up dexosomes, PKH67-labeled Exo-OVA and Exo-SIIN were cocultured with splenocytes from wild type mice. Interestingly, DCs and MZBs were shown to bind dexosomes most efficiently in vitro, although follicular B cells and MσBS also bound dexosomes but to a lesser extent (Fig. 5C). Kinetic in vivo experiments using BrdU showed that the CD4+ T cell–dependent activation of CD8+ T cells occurred early after Exo-OVA immunization (Fig. 4D, 4E), suggesting that early B cell–mediated mechanisms, such as Ag shuttling, are involved.

To investigate whether MZBs are involved in dexosome-mediated effects in vivo, we next immunized wild type and CD19−/− mice, which lack MZBs (20), with Exo-OVA and evaluated the OVA-specific CD8+ T cell response in the spleen 7 d postimmunization. CD19−/− mice had significantly lower frequencies of H-2Kb/SIINFEKL pentamer–positive cells (p < 0.05) (Fig. 5D), and significantly fewer cells were capable of producing IFN-γ (p < 0.05) (Fig. 5E) than in wild type mice, indicating that MZBs and/or complement-mediated regulation are

![FIGURE 4.](http://www.jimmunol.org/) Dexosomes loaded with OVA induce a CD8+ T cell response that is CD4+ T cell dependent. (A–C) Wild type (WT) and CD4 deficient (CD4−/−) mice were immunized with 40 μg Exo-OVA or Exo-SIIN. (A) Seven days postimmunization, the spleens were taken and analyzed for the presence of SIINFEKL-specific CD8+ T cells by H-2Kb/SIINFEKL-pentamer staining. (B) Splenocytes were stimulated with SIINFEKL peptide for 22 h in an IFN-γ ELISPOT to quantify SIINFEKL-specific IFN-γ–producing cells. (C) On day 6 after dexosome immunization, unloaded (CFSEhigh) and SIINFEKL–loaded (CFSElow) CD45.1+ splenocytes were injected at a 1:1 ratio. One day later, mice were euthanized, and spleens were analyzed for specific killing by flow cytometry and displayed as percentage specific lysis (%CFSEpeptide/%CFSEno peptide) × 100. (D and E) WT and CD4−/− mice were injected with 40 μg Exo-OVA and given BrdU from day 0 to 1, day 1 to 3, day 3 to 5, or day 5 to 7. On days 1, 3, 5, and 7, mice were euthanized, and splenocytes were analyzed for the number of BrdU-positive SIINFEKL–specific CD8+ T cells by BrdU and H-2Kb/SIINFEKL-pentamer staining (D) and by IFN-γ ELISPOT (E). Dots represent single mice, and data in (A–C) are pooled from three independent experiments with 3–10 mice/group. Data in (D) and (E) are pooled from two experiments with three mice/group. *p < 0.05, **p < 0.01, naive versus wild type mice, *p < 0.05, wild type versus CD4−/− mice, nonparametric two-tailed Mann–Whitney test.
involved in dexosome-induced immune responses. Interestingly, the OVA-specific Ab response was defective in CD19<sup>−/−</sup> mice both after one injection of dexosomes, as well as after boost injection with OVA protein (data not shown), suggesting a lack of B cell activation after dexosome immunization in mice lacking MZBs.

**Mice immunized with Exo-OVA survive longer after B16/OVA tumor challenge**

Finally, we evaluated the capacity of dexosomes in a B16 tumor-challenge model. Wild type mice were immunized with either Exo-OVA or Exo-SIIN via single (day 14) or repeated (days 0 and 14) immunizations before injection of OVA-expressing B16 tumor cells on day 21. All mice receiving dexosomes had a significantly increased median survival in comparison with naive mice, and Exo-OVA–immunized groups had a significantly longer median survival compared with Exo-SIIN–immunized groups (p < 0.001). A boost with either Exo-SIIN or Exo-OVA did not affect survival compared with single-immunized mice (Fig. 6A). Both groups of Exo-OVA–immunized mice also displayed a slower tumor growth than did the corresponding Exo-SIIN–immunized groups (Fig. 6B). Upon euthanization of the tumor-bearing mice, we detected significantly stronger SIINFEKL–specific CD8<sup>+</sup> T cell responses in the Exo-OVA–immunized groups, but not in the Exo-SIIN immunized groups, compared with naive mice, both in the spleen (Fig. 6C, 6D) and tumor (Fig. 6E, 6F). Moreover, Exo-OVA–immunized mice displayed significantly higher OVA-specific IgG Abs in comparison with naive mice (data not shown). In conclusion, dexosomes loaded with whole-OVA protein conferred better protection against an OVA-expressing tumor and induced a stronger systemic, as well as intratumoral OVA-specific, cytotoxic T cell response.

**Discussion**

Dexosomes are considered promising cancer vaccine candidates because they are capable of blocking tumor development in mice (2). However, to develop optimal treatment vehicles, the mechanisms for immune activation need to be elucidated. We previously showed that dexosome-induced CD4<sup>+</sup> T cell responses are B cell dependent (15). In the present study we investigated cell types involved in CD8<sup>+</sup> T cell activation after dexosome immunization. We could show that dexosomes loaded with whole protein were superior to peptide-loaded dexosomes in inducing an Ag-specific CD8<sup>+</sup> T cell response in vivo. Moreover, our data also clearly show that epitopes other than the SIINFEKL epitope are needed for the generation of a CD8<sup>+</sup> T cell response, because the presence of CD4<sup>+</sup> T cells was crucial. Interestingly, B cells, possibly by transport via MZBs and regulation of CD4<sup>+</sup> T cells, were also involved in dexosome-induced CD8<sup>+</sup> T cell responses. Finally, the protection against an OVA-expressing tumor was more efficient when whole-OVA protein was present on dexosomes.

Dexosomes express immune-stimulatory molecules, such as MHC class I, MHC class II, and costimulatory molecules, and are regarded as inert vehicles mimicking DCs (21). However, clinical trials using peptide-loaded dexosomes displayed tumor-specific CD8<sup>+</sup> T cell responses in only a few patients (12, 13). Interestingly, we found that B cells are involved in both CD4<sup>+</sup> and CD8<sup>+</sup> T cell generation after dexosome immunization, shown in Btk<sup>−/−</sup> and μMT mice, respectively (Ref. 15 and present study). Dexosomes loaded directly (15) with MHC class II peptide or indirectly (present study) with MHC class I peptide, but not with B cell epitopes, were not sufficient to elicit CD4<sup>+</sup> or CD8<sup>+</sup> T cell responses, respectively. Thus, we speculate that dexosomes are dependent on B cell transport and B cell epitopes to induce optimal activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells. In accordance, in a B16 melanoma tumor model dependent on CD8<sup>+</sup> T cells for tumor clearance, tumor growth is enhanced by B cell depletion (22), showing the importance of B cells in tumor settings.

MZBs are known to shuttle blood-borne Ags and immune complexes from the blood and deposit their cargo onto FDCs in the spleen (19). In CD19<sup>−/−</sup> mice, which lack MZBs (20), we detected lower CD8<sup>+</sup> T cell responses. In line with this, CD19<sup>−/−</sup> mice did not produce OVA-specific Abs after Exo-OVA injection (data not shown), indicating that B cell activation after dexosome
FIGURE 6. Exo-OVA immunization induces antitumor immunity and increases survival in a B16/OVA melanoma model. Wild type mice were immunized with Exo-OVA or Exo-SIIN in single (day 14) and repeated (day 0 and 14) injections and challenged with B16 tumor cells expressing OVA protein on day 21. Mice were euthanized when tumors reached 1000 mm³ in volume. (A) Survival of the mice displayed in a Kaplan–Meier survival curve. (B) Tumor size for all groups at each time point. SIINFEKL-specific CD8+ T cells were measured by H-2Kb/SIINFEKL-pentamer staining in spleen (C) and tumor (D). Splenocytes (D) or tumor single cell cultures (F) were stimulated with SIINFEKL peptide for 22 h in an IFN-γ ELISPOT to quantify SIINFEKL-specific IFN-γ-producing cells. If no tumor was detected, mice were euthanized on day 54 or 63 (n = 2, Exo-OVA groups). Dots represent single mice, and data are pooled from two experiments with four mice/group. *p < 0.05, **p < 0.01, ***p < 0.0001, nonparametric two-tailed Mann–Whitney analysis (A, C–F), Kruskal–Wallis and Dunn multiple comparison test (B).

injection was lost in mice lacking MZBs. Hence, we suggest that MZBs are involved in shuttling of dexosomes from the blood to FDCs, leading to activation of B cells and CD4+ T cells. The transport of dexosomes by MZBs might be mediated by complement, because MZBs are involved in complement receptor CD21-dependent transport of immune complexes (23), and several complement factors were shown to be present on dexosomes (24). In accordance, dexosome transport by MZBs is plausible because we find that noncognate MZBs readily bind dexosomes (Fig. 5C). However, CD19−/− mice also have abnormalities in marginal zone DC and MΦ function (23), and we cannot exclude the involvement of these mechanisms.

Because the CD8+ T cell response was totally absent in CD4−/− mice, we propose that the role of B cells in cytotoxic T cell activation is primarily to activate CD4+ T cells. Because DCS in the spleen readily interact with dexosomes (Fig. 5C), at least in vitro, we propose that dexosomes are partially taken up by DCS in secondary lymphoid organs, leading to an activation of Ag-specific CD4+ T cells and CD8+ T cells. The T cell response is further enhanced by B cells activated by dexosomes deposited on FDCs by MZBs and B cell–CD4+ T cell interactions at the border of the T cell/B cell zone (25). Further studies using BCR-transgenic mice will dissect the importance of cognate B cell–T cell interactions in the dexosome-induced immune response.

We showed previously that dexosomes produced by OVA-pulsed DCs carry whole-OVA Ag (15) where at least parts of the OVA protein seem to be accessible for B cell recognition. However, how the OVA protein is bound to dexosomes is still not understood. Because we can detect whole OVA on dexosomes by ELISA and flow cytometry (S. Hiltbrunner, U. Gehrmann, T.I. Näslund, and S. Gabrielson, unpublished observations), the protein is either inserted into the dexosomal membrane or attached to the outer surface of the dexosome by binding to membrane receptors. Preliminary data indicate that the majority of OVA protein is located within the dexosomal membrane, where it is protected from protease degradation, and is not bound to receptors on the dexosomes (S. Hiltbrunner, U. Gehrmann, T.I. Näslund, and S. Gabrielson, unpublished observations). This indicates that rerouting of intact Ag into the dexosomal membrane takes place in DCs. Moreover, we also detect whole hen egg lysozyme protein on dexosomes released by hen egg lysozyme–pulsed DCs. This suggests that Ag rerouting is a general property of Ags and is not specific to the OVA protein (S. Hiltbrunner, U. Gehrmann, T.I. Näslund, and S. Gabrielson, unpublished observations). These data suggest that most proteins, also intact tumor Ags, can be loaded onto dexosomes.

Based on our findings, the inclusion of cancer-associated B cell epitopes in future dexosome-based clinical trials should be considered a way to improve the immunogenicity of dexosomes to boost CD8+ T cell responses. Dexosomes are attractive vaccine or treatment vehicles because they carry molecules capable of stimulating a broad repertoire of the immune system (26, 27). Moreover, dexosomes were shown to be superior to DCs in preventing tumor growth in mouse models (2), an effect attributed to their inert properties. In conclusion, we showed that functional CD8+ T cell responses are induced after dexosome immunization in vivo where both Th cells and B cells were shown to be needed for optimal activation and protection against tumor growth. Future studies will elucidate whether the addition of other ligands on the dexosomes could further potentiate immune responses.

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Disclosures

The authors have no financial conflicts of interest.
References