Synergistic Induction of Adaptive Antitumor Immunity by Codelivery of Antigen with α-Galactosylceramide on Exosomes

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Abstract
Exosomes and the invariant NKT (iNKT) immune cell ligand α-galactosylceramide (αGC) may offer novel tools for cancer immunotherapy. In this study, we investigated whether exosomes loaded with αGC can activate iNKT cells and potentiate a cancer-specific adaptive immune response. αGC loaded exosomes readily activated iNKT cells both in vitro and in vivo. Exosomes loaded with αGC plus the model antigen ovalbumin (OVA) induced potent NK and γδ T-cell innate immune responses, and they also synergistically amplified T- and B-cell responses that were OVA specific. In contrast to soluble αGC, which anergizes iNKT cells, we found that αGC/OVA-loaded exosomes did not induce iNKT cell anergy but were more potent than soluble αGC + OVA in inducing adaptive immune responses. In an OVA-expressing mouse model of melanoma, treatment of tumor-bearing mice with αGC/OVA-loaded exosomes decreased tumor growth, increased antigen-specific CD8⁺ T-cell tumor infiltration, and increased median survival, relative to control mice immunized with soluble αGC + OVA alone. Notably, an additional injection of αGC/OVA-loaded exosomes further augmented the treatment effects. Our findings show that exosomes loaded with protein antigen and αGC will activate adaptive immunity in the absence of triggering iNKT-cell anergy, supporting their application in the design of a broad variety of cancer immunotherapy trials. Cancer Res; 73(13); 3865–76. ©2013 AACR.

Introduction
Effective antitumor immunity needs activation of both the innate and adaptive immune system to overcome the immune evasion strategies used by tumors. Furthermore, a long-lasting adaptive immune response needs a boost by the innate immune system (1). Natural killer (NK) cells, cytotoxic CD8⁺ T cells, and γδ T cells can induce cell death, whereas antibodies are important for antibody-dependent cellular cytotoxicity (ADCC; ref. 2). Thus, effective antitumor therapy needs to activate multiple players of the immune system to mount an effective, multifaceted, and long-lasting immune response (3).

Exosomes are small membrane vesicles around 100 nm, derived from the endosomal compartment and are secreted by many different cell types, among other cancer cells and dendritic cells (4). They can express immunostimulatory molecules such as MHC molecules, CD80 and CD86 (5) and elicit antigen-specific CD4⁺ (6), CD8⁺ T cell (7), B-cell responses (8), and NK-cell (9) responses in vivo. Exosomes from melanoma peptide-pulsed dendritic cells were able to reverse tumor growth in mice (7), which led to the evaluation of exosomes as therapeutic agents and vaccine vehicles (10). However, two phase I clinical trials using peptide-loaded dendritic cell exosomes in patients with melanoma (11) and non–small cell lung cancer (12) showed that exosomes were well tolerated but had limited immunostimulatory effects. Thus, a better understanding of the exosomal immune response is needed to increase exosomal immunogenicity and thereby improving the chances of therapeutic success. We have recently shown that exosomes induce CD4⁺ and CD8⁺ T-cell responses in a B-cell–dependent manner in vivo (13, 14) where protein loading, but not peptide loading of exosomes enhanced their immunogenicity. We now asked whether dendritic cell–derived exosomes, carrying the lipid antigen–presenting molecule CD1d (15, 16), can be loaded with glycolipid antigen to activate invariant NKT (iNKT) cells, a T-cell subset that has been shown to be important for anticancer responses (17) and whether this would boost T- and B-cell responses to a protein antigen on the same exosome.

iNKT cells are a cell type that share characteristics with both innate and adaptive immune cells (18), which recognizes self and bacterial glycolipids in a CD1d-dependent manner (19). Upon activation, iNKT cells rapidly release cytokines such as IFN-γ and interleukin (IL)-4 (19) and have impact on
subsequent NK-, T- and B-cell responses (20–22). Alpha-galactosylceramide (αGC) is a glycolipid that induces a rapid activation of inKT cells in vivo (19). However, injection of soluble αGC causes anergy of inKT cells (23) and multiple injections of αGC in humans have had limited therapeutic effects (24). Because coupling of αGC-loaded CD1d molecules to antigenic protein or nanoparticles (25) was suggested to overcome anergy induction (26), we speculated that dendritic cell–derived exosomes could serve as an endogenous delivery platform for protein and glycolipid antigens without inducing anergy.

We report that exosomes loaded with αGC and ovalbumin (OVA) activate inKT cells, overcome anergy induction, and amplify tumor-specific adaptive immune responses with implications for the development of novel cancer immunotherapy.

Materials and Methods

**Mice and antibodies**

C57Bl/6, Vc14-Ccr7−/− (kindly donated by Dr. G. Berne, Karolinska Institutet), and CD1d−/− mice (kindly donated by Prof. K. Kärre, Karolinska Institutet) were bred and maintained under pathogen-free conditions at Karolinska Institutet’s animal facility. All experiments were approved by the Stockholm Regional Ethics Committee. A list of antibodies used is available in Supplementary Table S1.

**Bone marrow–derived dendritic cells**

Bone marrow cells were prepared from female C57Bl/6 or CD1d−/− mice as previously described (13). On day 6, cells were incubated overnight with 300 μg/mL OVA (grade V; Sigma), 2 μg/mL SIINFEKL (Innovagen), and/or 100 ng/mL αGC (KRN-7000; Larodan Fine Chemicals). On day 7, supernatants were discarded and cells were grown in new medium containing exosome-depleted FCS (27), GM-CSF, IL-4, and 30 ng/mL LPS (Sigma). On day 9, supernatants were used for exosome preparation.

**Exosome preparation**

Exosomes were prepared as described before (27), with some modifications. After centrifugation for 30 minutes at 3,000 × g, supernatants were filtered through a 0.22-μm cutoff filter (Nordic Biosite), pelleted and washed in PBS at 100,000 × g for 2 hours and 10 minutes. The final pellet was re-suspended in a small volume of PBS and protein content was measured using a DC protein assay according to the manufacturer’s instructions (Bio-Rad). Exosomes were aliquoted and frozen at −80°C.

**Exosome phenotyping**

Sulfate–aldehyde latex microspheres (4 μm; Invitrogen) were coated with anti-CD9 (BD Biosciences) antibodies to enrich for exosomes on the beads as previously described (28). Exosomes from wt or CD1d−/− mice were coated onto anti-CD9 latex beads at a ratio of 50 μg protein per μL beads and phenotyped as described before (13) using specific antibodies and corresponding isotype controls (Supplementary Table S1). Data were acquired using a FACSCalibur (BD Biosciences) and analysis was done using FlowJo software (Tree Star Inc.).

**In vitro proliferation**

A total of 7.5 × 10⁵ splenocytes were labeled with 5 μmol/L carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) and stimulated with exosomes at different concentrations (0.05, 0.5, 5, and 50 μg/mL) for 72 hours. DimerX (BD Biosciences) was loaded overnight with sonicated αGC at 37°C and labeled with antimouse IgG, Alexa 647 (Invitrogen). To stain for inKT cells, cells were Fc−-blocked (BD Biosciences), incubated with Live/Dead viability marker (Invitrogen), DimerX, and antibodies against B220, CD4, NK1.1, T-cell receptor β chain (TCR-β; all Biolegend). Data were acquired using a FACS Aria (BD Biosciences).

**In vivo proliferation**

C57Bl/6 wild-type mice (wt) or CD1d−/− female mice were injected on day 0 or on days 0 and 14 i.v. with soluble αGC, soluble OVA, or 40 μg of exosomes from wt or CD1d−/− dendritic cells. Mice were fed with 0.8 mg/mL 5-bromo-2'-deoxyuridine (BrdU; Sigma) in drinking water either for 7 days or in intervals from day 0 to 1, day 1 to 3, day 3 to 5, or day 5 to 7. Mice were sacrificed on days 1, 3, 5, 7, or 21 and blood, liver, and spleen were removed. Hepatic lymphocyte preparations and splenocyte single-cell suspensions were prepared as described previously (13, 29) and serum was prepared from coagulated blood and frozen at −20°C. BrdU incorporation was measured using a BrdU staining kit (BD Biosciences), according to the manufacturer’s instructions. For OVA-specific CD8+ T-cell staining, cells were incubated with phycoerythrin (PE)-labeled H-2Kb/SIINFEKL pentamer (ProImmune). Data were acquired using FACS Aria (BD Biosciences).

**B16/OVA melanoma tumor model**

A total of 1 × 10⁶ B16/OVA melanoma cells were injected s.c. in the right flank of C57Bl/6 mice. Mice were treated i.v. either with PBS or 40 μg of exosomes 11 days after tumor injection, when tumors were palpable, or on day 4, or on day 4 and 11 as indicated in figure legends. Tumor growth was monitored regularly and mice were euthanized when tumors reached 1,000 mm³ in size. Tumors were removed, and either embedded in Killick (Bio-Optica) and frozen at −80°C for immunohistochemistry or homogenized for FACS analysis using antibodies against CD45, B220, TCR-β, CD8 (Supplementary Table S1) and PE-labeled H-2Kb+/SIINFEKL pentamer (ProImmune).

**Tumor immunohistochemistry**

Eight-micrometer sections of Killick-embedded tumors were fixed in acetone, dried overnight, and blocked with goat serum, avidin, and biotin (Vector Laboratories). Samples were stained with anti-TCR-β-APC and analyzed using a Leica DM IRBE microscope.

**Intracellular cytokine staining**

Splenocytes were restimulated ex vivo for 4 hours using 50 ng/mL Phorbol 12-myristate 13-acetate (PMA), 500 ng/mL...
ionomycin, and 1 μg/mL Brefeldin A (all from Sigma) as previously described (30).

**ELISPOT**

Enzyme-linked immunospot assay (ELISPOT) for IFN-γ was done according to the manufacturer’s instructions (Mabtech) using 200,000 splenocytes per well. Splenocytes from Vα14-transgenic mice were stimulated with a serial dilution of soluble αGC or 0.05, 0.5, 5, and 50 μg/mL of exosomes for 72 hours. Splenocytes were stimulated in vitro with 2 μg/mL αGC, 2 μg/mL SIINFEKL, 2 μg/mL OVA323-339 peptide (Innovagen), 2 μg/mL Concanavalin A (Sigma), or left unstimulated for 22 hours.

ELISA

IFN-γ and IL-17A concentrations were determined in serum or in supernatants from ELISPOT experiments using ELISA according to the manufacturer’s instructions (Mabtech). Mouse sera from *in vivo* experiments were analyzed for concentrations of OVA-specific IgG, total IgG, IgG2c, and IgG1 as described before (13).

**Statistical analysis**

Student *t* test or one-way ANOVA with Bonferroni’s correction was used for normally distributed data. Mann–Whitney or Kruskal–Wallis with Dunn’s correction was used for nonparametric data. For kinetic experiments, 2-way ANOVA with Bonferroni’s correction was used. Statistical analysis was conducted using GraphPad software (GraphPad Inc.).

**Results**

**Dendritic cell–derived exosomes express CD1d and induce iNKT-cell activation in vitro**

To confirm that exosomes from bone marrow–derived dendritic cells express CD1d (16), exosomes from C57Bl/6 wt or CD1d<sup>−/−</sup> dendritic cells were generated and phenotyped using anti–CD1d–coated latex beads and flow cytometry. Both wt and CD1d<sup>−/−</sup> exosomes expressed similar amounts of MHC class II (I<sup>A</sup>/I<sup>E</sup>), CD9, CD11c, CD40, CD54, CD80, CD81, and CD86, but only exosomes from wt dendritic cells expressed CD1d ( *P* = 0.004; Fig. 1A).

Next, we investigated whether exosomes loaded with αGC could activate iNKT cells *in vitro*. OVA-loaded (Exo-OVA) or αGC-loaded exosomes (Exo-αGC) from wt mice or Exo-αGC from CD1d<sup>−/−</sup> mice (CD1d<sup>−/−</sup> Exo-αGC) were added to splenocytes from Vα14 mice, transgenic for the iNKT cell receptor. Exo-αGC induced proliferation in the majority of iNKT cells whereas CD1d<sup>−/−</sup> Exo-αGC induced substantially less iNKT cell proliferation and Exo-OVA induced none (Fig. 1B and C) shown by CFSE dilution assay. Exo-αGC also induced increased numbers of IL-4–producing splenocytes as determined by ELISPOT (Fig. 1D) and higher levels of IFN-γ and IL-17A using ELISA than did CD1d<sup>−/−</sup> Exo-αGC and Exo-OVA (Fig. 1E and F). However, CD1d<sup>−/−</sup> Exo-αGC induced higher cytokine responses than Exo-OVA (Fig. 1D–F), indicating that αGC is not exclusively loaded onto CD1d molecules in exosomes. Together, these results suggest that exosomes from αGC-pulsed dendritic cells can induce potent iNKT cell proliferation and cytokine production mainly, but not exclusively, via exosomal CD1d *in vitro*.

**Exosomes loaded with αGC activate iNKT, NK, and γδ T cells *in vivo***

Next, we investigated the effect of exosomes loaded with αGC and the model antigen OVA [Exo(αGC-OVA)] or the CD8<sup>+</sup> T-cell OVA-specific peptide SIINFEKL [Exo(αGC-SIINFEKL)] *in vivo*. We injected 40 μg of Exo-OVA, Exo-SIINFEKL, Exo(αGC-SIINFEKL), Exo(αGC-OVA), or PBS i.v. into wt recipient mice, which were fed with BrdU for 7 days. Splenic iNKT cells proliferated in response to Exo(αGC-OVA) and Exo(αGC-SIINFEKL) but not to Exo-OVA, Exo-SIINFEKL, and PBS as determined by flow cytometry (Fig. 2A and B). iNKT cells upregulated the activation marker CD69 on day 1 (Fig. 2C) and proliferated up to day 5 (Fig. 2D) in response to Exo(αGC-OVA), but not to Exo-OVA or PBS. Hepatic iNKT cells showed similar patterns of activation and proliferation as their splenic counterparts (data not shown). Intracutaneous cytokine staining showed that splenic iNKT cells produce IFN-γ during the first 5 days (Fig. 2E), whereas IL-4 was produced during the first 3 days (Fig. 2F) in response to Exo(αGC-OVA) but not to PBS or Exo-OVA.

As reported by others (20, 31), activation of iNKT cells led to an early activation and proliferation of dendritic cells, NK, and γδ T cells, the latter two being innate-like lymphocytes with important functions in anticancer immunity (Supplementary Fig. S1, data not shown). Interestingly, we also detected significantly lower levels of the complement receptor CD21 on marginal zone B (MZB) cells on day 1 after Exo(αGC-OVA) injection and increased proliferation between days 1 and 3 in comparison to Exo-OVA immunization (Supplementary Fig. S2A and S2B). These data indicate that αGC-loaded exosomes induce an early iNKT-cell response, dendritic cell, MZB cell activation as well as NK- and γδ T-cell activation and proliferation *in vivo*.

**Exosomes loaded with αGC potentiate OVA-specific CD8<sup>+</sup> T-cell responses**

CD8<sup>+</sup> T cells are adaptive immune cells that are crucial for exosome-induced antitumor immunity (7). We detected that Exo(αGC-OVA) stimulated OVA-specific CD8<sup>+</sup> T-cell proliferation and led to a larger pool of OVA-specific CD8<sup>+</sup> T cells, 7 days after exosome injection in comparison to PBS and Exo-OVA–immunized groups (Fig. 3A–C). This effect was not due to an αGC-induced polyclonal CD8<sup>+</sup> T-cell expansion because Exo(αGC-SIINFEKL) immunized mice did not have increased numbers of OVA-specific CD8<sup>+</sup> T cells (Fig. 3B). This is in agreement with previous data where SIINFEKL peptide-loaded exosomes are not sufficient to stimulate proliferation of OVA-specific CD8<sup>+</sup> T cells *in vivo*, because B-cell activation is needed for exosome-induced T-cell activation *in vivo* (14). The increase in OVA-specific CD8<sup>+</sup> T cells was iNKT cell dependent (Fig. 3C). Proliferation of OVA-specific CD8<sup>+</sup> T cells was detected on days 5 and 7 in Exo(αGC-OVA)-immunized mice (Fig. 3D), leading to a significantly increased OVA-specific CD8<sup>+</sup> T-cell pool on days 5.
Exosomes from bone marrow–derived dendritic cells loaded with αGC activate iNKT cells in vitro. A, exosomes from dendritic cell of wt mice (wt) or CD1d−/− mice (CD1d−−) loaded with αGC and/or OVA were coupled to anti-CD9–coated latex beads and analyzed for surface molecule expression using flow cytometry. Data are from 20 (wt) or 15 (CD1d−−) exosome batches. Data are expressed as the MFI ratio between specific antibody and corresponding isotype control. Statistical analysis was done using nonparametrical Mann–Whitney test. B, representative CFSE dilution histogram plots and quantification of CFSE dilution assay using splenocytes from Vπ14 transgenic mice stimulated with different concentrations of exosomes from OVA-pulsed wt dendritic cell (Exo-OVA) or from αGC-pulsed wt dendritic cell (Exo-αGC) or CD1d−− dendritic cell (CD1d−− Exo-αGC). C, numbers indicate the percentage of proliferated iNKT cells, defined as B220−, TCR-β+ , and DimerX+ cells. Data are from 3 independent experiments using duplicates in each experiment. D, Vπ14 splenocytes were stimulated with increasing amounts of exosomes in an IL-4 ELISPOT assay for 72 hours. E and F, supernatants from ELISPOT experiments were analyzed for IFN-γ and IL-17A using ELISA. Data are pooled from 3 independent (D) or 5 independent experiments (D-F). Two-way ANOVA was used to test for statistical significance. Bars indicate mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

and 7 when compared with PBS and Exo-OVA (Fig. 3E). Similar kinetics for OVA-specific CD8+ T cells were observed in the liver (data not shown). Exo(αGC-OVA) immunization generated increased numbers of SIINFEKL–specific IFN-γ producing cells as detected by ELISPOT (Fig. 3F), an effect that was dependent on exosomal CD1d (Fig. 3G). These results show that αGC-loaded exosomes amplify antigen-specific CD8+ T-cell responses via iNKT cells in vivo and that exosomal CD1d has an important role in that response.

αGC-loaded exosomes boost CD4+ T- and B-cell responses

Antibodies are crucial in initiating ADCC, which is an important mechanism in antitumor immunity. Thus, we investigated the effect of αGC-loaded exosomes on T-helper cell responses and humoral immunity. We observed that proliferation of CD4+ T cells was significantly increased in the spleen in response to Exo(αGC-OVA) compared with PBS and Exo-OVA 7 days after exosome injection (Fig. 4A) and that this effect
**αGC on Exosomes Amplifies Antitumor Immunity**

Figure 2. Exosomes loaded with αGC activate iNKT cells in vivo. C57Bl/6 mice were injected i.v. with PBS or 40 μg Exo-OVA, Exo(αGC-OVA) or with exosomes from SIINFEKL (Exo−SIINFEKL) or αGC and SIINFEKL [Exo(αGC−SIINFEKL)] pulsed dendritic cells and fed BrdU in drinking water for 7 days and euthanized on day 7 (A, B) or for 1 to 2 days on day 0, 1, 3, or 5 and euthanized on day 1, 3, 5, or 7 (C−E). A and B, representative BrdU-histogram plots (A) and proliferation of splenic iNKT cells (defined as BrdU+/CD4+/TCRβ+, DimerX+ live lymphocytes; B). Data are pooled from 4 independent experiments. One-way ANOVA with Bonferroni’s multiple comparison test was used to determine statistical significance. Dots represent single mice and lines indicate the mean ± SEM. C and D, expression of CD69 (C) or proliferation of splenic iNKT (D) cells as assessed by flow cytometry. Data are pooled from 2 independent experiments. Dots represent mean ± SEM. Two-way ANOVA with Bonferroni’s multiple comparison test was used to test for statistical significance. E, intracellular flow cytometry for IFN-γ and IL-4-expressing splenic iNKT cells after ex vivo restimulation with PMA/ionomycin/Brefeldin A for 4 hours. Data are pooled from 2 independent experiments. Dots represent mean ± SEM. Two-way ANOVA with Bonferroni’s multiple comparison test was used to test for statistical significance. **, P < 0.01; ***, P < 0.001 when compared with PBS group; ***, P < 0.001 when compared with the Exo-OVA group. For all experiments, at least 3 mice were used per group and time point.

was iNKT cell–dependent because responses were lower in CD1d−/− mice (Fig. 4B). Injection of Exo(αGC-OVA) also induced proliferation of follicular helper T cells (Tfh-cells; Supplementary Fig. S3), which are important for antibody class switching. In line with this, we detected increased numbers of total and proliferated germinal center B cells and plasma cells (Supplementary Fig. S4B−S4G), but not of follicular B cells (Supplementary Fig. S4A), and OVA-specific IgG levels were significantly higher already 7 days after immunization with Exo(αGC-OVA) when compared with all other groups (Fig. 4C). Intriguingly, only the Th1 antibody subclass IgG2a, but not IgG1 or total IgG antibodies, were significantly increased in serum of Exo(αGC-OVA)-treated mice compared with both PBS and Exo-OVA (Fig. 4D). Thus, Exo(αGC-OVA) induce CD4+ T-cell activation and increased B-cell responses, possibly via the induction of T-follicular helper cells and germinal center formation.

**Antigen-loaded exosomes are more potent than soluble antigens in inducing adaptive immunity**

It has been suggested that codelivery of αGC together with a protein antigen to the same APC is important to potentiate adaptive immune responses (32). To compare the efficiency of exosome-bound αGC and soluble αGC as adjuvant, we estimated the OVA and αGC content on Exo(αGC-OVA), using IFN-γ ELISpot and ELISA (Fig. 5A and B). We injected 40 μg of Exo(αGC-OVA) or the equivalent amount of soluble OVA (range: 32−320 ng) and αGC (range: 22−200 ng) and measured proliferation of innate and adaptive immune cells. Importantly, Exo(αGC-OVA) were less potent in inducing
iNKT- and NK-cell proliferation compared with soluble αGC and OVA, whereas the opposite was true for γδ T cells, CD4⁺ T cells, and OVA-specific CD8⁺ T cells (Fig. 5C). After a second injection, we also observed stronger B-cell responses with significantly higher numbers of GC B cells and OVA-specific IgG levels in exosome-treated mice (Fig. 5D). These findings show that αGC is a more effective adjuvant for adaptive immune responses when loaded to exosomes compared to soluble ligand.

Exo(αGC-OVA) does not induce iNKT-cell anergy

Soluble αGC induces iNKT-cell anergy already after 1 injection, a possible reason for the limited clinical success of αGC-based immunotherapies (23, 25). To test whether αGC-loaded exosomes were less prone to induce iNKT-cell anergy, we injected PBS, soluble αGC + OVA, or Exo(αGC-OVA) 2 times with a 2-week interval (Fig. 6A) and measured serum IFN-γ, iNKT-cell proliferation, and IFN-γ production in ELISPOT to determine anergy induction. As expected, soluble αGC and

Figure 3. αGC on exosomes increases OVA-specific CD8⁺ T-cell responses via iNKT cells. C57Bl/6 mice were injected i.v. with PBS or 40 μg Exo-OVA, Exo(αGC-OVA), Exo-SIINFEKL, or Exo(αGC-SIINFEKL) and fed BrdU in drinking water for 7 days and euthanized on day 7 (A, B, C, F, G) or 1 to 2 days on day 0, 1, 3, or 5 and euthanized on day 1, 3, 5, or 7 (D, E). A and B, representative dot plots (A) and quantification of splenic OVA-Pentamer⁺ CD8⁺ T cells (defined as OVA-Pentamer⁺ of B220⁻, CD3⁻, CD8⁻ live lymphocytes; B). Data are pooled from 4 experiments and one-way ANOVA with Bonferroni’s multiple comparison test was used to determine statistical significance. Dots represent single mice and lines mean ± SEM. C, percentage of OVA-specific CD8⁺ T cells in wt and CD1d⁻/⁻ mice in response to Exo-OVA and Exo(αGC-OVA). Data are from 3 independent experiments and Student t test was used to test for significance. Dots represent single mice and lines mean ± SEM. D and E, proliferation (defined as BrdU⁺ cells; D) and percentage (E) of splenic OVA-Pentamer⁺ CD8⁺ T cells. Data are pooled from 2 independent experiments. Two-way ANOVA with Bonferroni’s multiple comparison test was used to test for statistical significance. Dots represent mean ± SEM. F, IFN-γ ELISPOT after ex vivo stimulation of splenocytes with SIINFEKL-peptide for 22 hours. Data are from 4 experiments and one-way ANOVA with Bonferroni’s multiple comparison test was used to determine statistical significance. Dots represent single mice and lines mean ± SEM. G, IFN-γ ELISPOT of splenocytes from wt mice in response to Exo-OVA and Exo(αGC-OVA) from wt and CD1d⁻/⁻ mice after ex vivo restimulation with SIINFEKL-peptide for 22 hours. Data are from 2 independent experiments and Student t test was used to test for significance. Dots represent single mice and lines mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001 when compared with PBS group; *, P < 0.05; ***, P < 0.001 when compared with the Exo-OVA group. For all experiments, at least 3 mice were used per group and time point.
OVA induced significantly higher levels of serum IFN-γ after the first injection when compared with both PBS and Exo(αGC-OVA) groups. However, only Exo(αGC-OVA) induced increased levels of serum IFN-γ after both injections (Fig. 6B). After boosting, iNKT cells from soluble αGC + OVA, but not from Exo(αGC-OVA)-injected mice had decreased CD69 expression (Fig. 6C) and were refractory to ex vivo restimulation with αGC (Fig. 6D). Accordingly, we detected significantly more IFN-γ producing OVA-specific CD8+ T cells in splenocytes from twice exosome-injected mice (Fig. 6D). These results indicate that αGC-loaded exosomes can stimulate IFN-γ secretion by iNKT cells even after a second injection, in contrast to soluble αGC, and strikingly, boost adaptive immune responses.

**Exo(αGC-OVA) decrease tumor growth and induce T-cell infiltration in a mouse melanoma model**

Having shown that exosomes codelivering glycolipid and protein antigen potentiate adaptive immune responses without inducing iNKT-cell anergy, we investigated the potency of Exo(αGC-OVA) to improve antitumor immunity. We injected mice with OVA-expressing B16 melanoma cells and treated on day 4 and/or 11 with PBS, Exo-OVA, soluble αGC + OVA, or Exo(αGC-OVA). Tumor bearing mice receiving 1 (day 4 or day 11) or 2 (day 4 and 11) injections of Exo(αGC-OVA) had a significantly increased median survival compared with PBS, Exo-OVA, and soluble αGC + OVA groups (Fig. 7A and B; Supplementary Fig. S5A and S5B). Moreover, 2 injections of Exo(αGC-OVA) significantly prolonged survival while slowing down tumor growth when compared with 1 injection of exosomes (Fig. 7A and B). Using flow cytometry and immunofluorescence, we observed more tumor T-cell infiltrates and detected higher levels of OVA-specific CD8+ T cells in tumor tissues (Fig. 7C; Supplementary Fig. S5C). Similarly, OVA-specific IgG levels in serum of Exo(αGC-OVA)-treated mice compared with Exo-OVA or PBS-treated mice were significantly elevated (Fig. 7D; Supplementary Fig. S5D). Together these results show that Exo(αGC-OVA) but not soluble αGC + OVA induce tumor-specific B- and T-cell responses that lead to potent antitumor immunity and that treatment effects can be amplified by 2 injections of Exo(αGC-OVA).

**Discussion**

We show in this study that αGC-loaded exosomes can induce iNKT-cell activation in vitro and in vivo, without inducing iNKT-cell anergy, leading to enhanced adaptive immune responses and increased antitumor immunity. Importantly, only αGC codelivered with a protein antigen on exosomes boosted adaptive immune responses and could activate iNKT cells upon a second injection. These are important findings to consider when designing future cancer immunotherapy.

Exosomes from wt BMDC loaded with αGC induced stronger iNKT-cell proliferation and proinflammatory cytokine production than exosomes from CD1d<sup>−/−</sup> BMDC in vitro, arguing for an important role of exosomal CD1d. In vivo, CD1d<sup>−/−</sup> exosomes induced significantly lower numbers of IFN-γ producing OVA-specific CD8<sup>+</sup> T cells, supporting that exosomal CD1d is important for the induced adaptive immune response. However, our in vitro data suggest that CD1d<sup>−/−</sup> exosomes also carry αGC in a CD1d independent fashion,
perchased on uptake receptors such as LDL receptors (33). We and others reported previously that whole protein antigens can be retained on exosomes after dendritic cell pulsing (8, 13, 14) and lipid antigens might be retained on exosomes in a similar fashion. Alternatively, the αGC not loaded on CD1d could be located within the exosome. However, the mechanism of how externally added antigen is retained in membrane vesicles remains a matter of investigation.

In this study, we confirmed our recent finding that B-cell activation is needed for a potent CD8+ T-cell activation in vivo (14). Here, αGC loading of exosomes was not able to overcome the inability of peptide-loaded exosomes to induce antigen-specific CD8+ T-cell responses. We speculate that the adjuvant properties of αGC cannot compensate for the need of CD4+ and B-cell epitopes to induce cytotoxic T-cell responses. In fact, our data suggest that αGC enhances the involvement of dendritic cells and MZBs during the induction of the exosome-induced immune response. Early iNKT-cell activation coincided with upregulation of the costimulatory molecule CD86 on dendritic cells (26, 34, 35) and downregulation of the C3-binding complement receptor CD21 on MZBs. Dendritic cells are considered important cells for exosome-induced
T-cell activation (36) and our recent data suggest that also B cells, including MZB cells, have a role in exosome-induced immune responses (14, 37). It is possible that, similar to immune complexes (38), MZB-cell transport exosomal cargo into splenic B-cell follicles. Interestingly, C3 fragments have been found in proteomic analyses of dendritic cell derived exosomes preparations (39). Thus, we speculate that exosome transport by MZB cells allows for B-cell activation, whereas dendritic cell transport of exosomes into the T-cell zone allows for T-cell activation and that both mechanisms are crucial for potent exosome-mediated immune effects.

Exosomes also induced NK- and γδ T-cell proliferation and IFN-γ production in an iNKT-cell–dependent manner. γδ T cells express NKG2D, which can bind to ligand-expressing tumor cells (40) and due to their cytotoxic properties have been attributed important functions in melanoma immunity (41). In our study, proliferation and cytokine kinetics were similar for NK- and γδ T-cell activation by αGC-loaded exosomes. Although NK cells were also efficiently activated by soluble αGC, γδ T cells were not. In contrast, a recent report showed that a high dose of soluble αGC can induce γδ T-cell activation (42). These differences might be due to exosomes lowering the threshold for αGC-induced γδ T cell activation by synergistically providing additional activation signals, such as NKG2D ligands (9). In the same study, γδ T-cell activation also led to an increased CD8⁺ T-cell response (42), indicating that the αGC-induced innate immune response is needed for effective adaptive immunity to develop.

Following the innate immune response, we observed proliferation of OVA-specific, IFN-γ–producing CD8⁺ T cells after injection with Exo(αGC-OVA), an effect that was superior to that of exosomes lacking αGC. It has recently been shown that αGC-activated iNKT cells can induce CD8α⁺ dendritic cells to produce CCL-17, a CCR4 ligand that led to increased CD8⁺ T-cell recruitment to the activated dendritic cell (32). This suggests an important role for codelivery of protein and glycolipid antigen to the same dendritic cell and is in accordance with the iNKT-cell–dependent increase in antigen-specific CD8⁺ T-cell numbers we detect after Exo(αGC-OVA) immunization. The cross-talk between iNKT cells and dendritic cells then allows for a selective recruitment of CD8⁺ T cells to activated, protein antigen-presenting dendritic cells.
The CD8+ T-cell response was accompanied by proliferation of Thelper cells and production of OVA-specific antibodies. Interestingly, we found that T follicular helper cells, important in germinal center formation, were proliferating 5 days after Exo(αGC-OVA) injection, preceding an increase in numbers of germinal center B cells and plasma cells on day 7. In accordance with this, a second injection of Exo(αGC-OVA) led to higher numbers of GC B cells and boosted OVA-specific IgG.

We speculate that Exo(αGC-OVA) induces the formation of Th1 cells, which leads to subsequent formation of germinal centers in which germinal center B cells undergo affinity maturation, somatic hypermutation, and differentiate into plasma cells that produce antigen-specific antibodies. We found that Exo(αGC-OVA) predominantly induced IgG2a antibodies, indicative of a Th1 immune response. In accordance, two studies have previously reported that exosomes induce Th1 immunity (8, 13). The production of IFN-γ during the early phase of the immune responses by iNKT, NK, and γδ T cells might lead to the Th1-biased ensuing adaptive immune response.

An exciting finding is that αGC-loaded exosomes, in contrast to soluble αGC, do not induce iNKT-cell anergy.
after a second injection. In the initial studies, low doses of αGC (200 ng injected intraperitoneally) could induce iNKT-cell unresponsiveness up to 1 month after injection. In our study, 100 ng of soluble αGC injected i.v. induced a remarkable unresponsiveness of iNKT cells 2 weeks after injection whereas exosomes maintained their ability to stimulate cytokine responses. This is important for the ensuing adaptive immunity because our data show that the OVA-specific CD8+ T-cell response persists after 2 injections of Exo(αGC-OVA), but not of soluble αGC and OVA.

To test whether this lack of anergy induction had a functional relevance for the induction of antitumor immunity, we evaluated the therapeutic effect of Exo(αGC-OVA) treatment in an OVA-expressing melanoma model. We observed additive treatment effects with two injections of Exo(αGC-OVA), suggesting that exosomal delivery might allow for multiple boost injections of αGC during a treatment regimen. Based on the results of this study, we speculate that the codelivery of lipid and a protein antigen is beneficial to induce adaptive antitumor immunity.

In summary, we present novel data stating that αGC-loaded exosomes boost innate and antigen-specific Th1 adaptive immunity and enhance antitumor immunity without inducing iNKT-cell anergy. Our approach increased the immunogenicity of dendritic cell–derived exosomes and should be taken into account when designing future clinical therapies for malignant diseases.

References


Synergistic Induction of Adaptive Antitumor Immunity by Codelivery of Antigen with α-Galactosylceramide on Exosomes

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