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Activation of invariant Natural Killer T lymphocytes in response to the α-galactosylceramide analogue KRN7000 encapsulated in PLGA-based nanoparticles and microparticles

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ABSTRACT

Invariant Natural Killer T (iNKT) cells have potent immunostimulatory activities that could be exploited for human therapies. The high-affinity CD1d antigen α-galactosylceramide analogue KRN7000 (KRN) activates a cascade of anti-tumor effector cells and clinical studies have already had some initial success. To improve the efficacy of the treatment, strategies that aim to vectorize KRN would be valuable. In this study, we intended to characterize and compare the effect of KRN encapsulated in poly(lactic-*co*-glycolic acid) (PLGA)-based nanoparticles (NPs, 90 nm) and microparticles instead of macroparticles (MPs, 715 nm) on the iNKT cell response. Our data show that whatever the size of the particles, vectorized KRN induced potent primary activation of iNKT cells *in vitro* and *in vivo*. We show that endocytosis of PLGA-based particles by dendritic cells is mediated by a clathrin-dependent manner and that this event is important to stimulate iNKT cells. Finally, we report that KRN vectorized in NPs and MPs exhibited different behaviours *in vivo* in terms of iNKT cell expansion and responsiveness to a recall stimulation. Collectively, our data validate the concept that KRN encapsulated in PLGA-based particles can be used as delivery systems to activate iNKT cells *in vitro* and *in vivo*.

1. Introduction

Invariant Natural Killer T (iNKT) cells represent an emerging population of "innate-like" immune cells expressing NK lineage receptors and an invariant T cell receptor (TCR)α chain (Vα14–Jα18 rearrangement in mice and Vα24–Jα18 rearrangement in humans) that pairs with a limited number of Vβ chains (for review, Bendelac et al., 2007). This cell population recognizes (glyco)lipid antigens (Ag) presented by the CD1d molecule expressed by antigen (Ag) presenting cells (APCs), including dendritic cells (DCs) (Bendelac et al., 2007; Cohen et al., 2009; Godfrey and Kronenberg, 2004; Taniguchi et al., 2003; Tupin et al., 2007; Van Kaer and Joyce, 2005). The role of iNKT cells in anti-tumoral responses was first appreciated with the discovery of α-galactosylceramide (α-GalCer), a glycosphingolipid originally isolated from a marine sponge (*Agelas mauritianus*) during anti-tumor compound screening from natural products (Kawano et al., 1997). In response to α-GalCer, iNKT cells rapidly and vigorously produce a wide array of cytokines that in turn initiate innate and adaptive immune responses (for reviews,

Abbreviations: iNKT cells, invariant Natural Killer T cells; TCR, T cell receptor; Ag, antigen; APCs, Ag presenting cells; DCs, dendritic cells; α-GalCer, α-galactosylceramide; PLGA, poly(D,L lactic-*co*-glycolic acid); NP, nanoparticle; MP, microparticle; MNCs, mononuclear cells; BM, bone marrow; PLA, poly-lactic acid.

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Bendelac et al., 2007; Cerundolo et al., 2009; Tupin et al., 2007; Van Kaer and Joyce, 2005). Through this activation cascade, α-GalCer exerts potent anti-tumoral and adjuvant activities *in vivo*, rendering it a powerful candidate for adjuvant therapy in cancer (for reviews, Fujii et al., 2007; Hayakawa et al., 2004; Molling et al., 2008; Taniguchi et al., 2010). Based on these encouraging results, attempts to exploit the anti-tumor property of free, non-vectorized, KRN have been made in the human system. Although the drug was well tolerated, no or moderate clinical responses were observed among the patients repeatedly inoculated with α-GalCer (Dhodapkar et al., 2003; Giaccone et al., 2002; Molling et al., 2005; Motohashi et al., 2002; van der Vliet et al., 2004). One potential explanation for this disappointing observation may lie in the low responsiveness of iNKT cells in patients. Moreover, as observed in the mouse system (Fujii et al., 2002; Matsuda et al., 2003; Parekh et al., 2005; Singh et al., 1999), α-GalCer induced a long-term anergy of human iNKT cells, thus preventing cytokine release upon a recall stimulation. These phenomena may be due, at least in part, to a lack of adequate KRN delivery systems to optimize the iNKT cell response. It is likely that formulation of KRN into particles will modify its bioavailability, thus modifying the nature, strength and duration of the iNKT cell response. Surprisingly enough, the use of formulations to improve the activities of KRN has so far received relatively little attention and assays have mainly been based upon liposomal formulations (Benoit et al., 2007; Tamura et al., 2008). In the present report, we used the biocompatible polymer poly(D,L lactic-*co*-glycolic acid) (PLGA) to vectorize the synthetic α-GalCer analogue KRN7000 (Morita et al., 1995). PLGA-based particles present several advantages over other delivery systems including their current use in clinical practice, their stability and biodegradability, and their ability to solubilize and control the *in vivo* release of lipophilic molecules (Dechy-Cabaret et al., 2004; Fahmy et al., 2008; Mundargi et al., 2008; Singh and O'Hagan, 2002). Since the size of the particles influences the outcome of immune responses in many systems (Cruz et al., 2010; Eldridge et al., 1991; Fifis et al., 2004; Hagenaars et al., 2008; Manolova et al., 2008; Reddy et al., 2007), we herein intended to characterize and compare the effect of KRN encapsulated into PLGA-based nanoparticles (NPs) and microparticles (MPs) on the iNKT cell response in the mouse system. Our data show that KRN vectorized in PLGA-based NPs and in MPs is bioavailable and activates iNKT cells both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Reagents and Abs

KRN7000 (2S,3S,4R)-1-O-(alpha-D-galactopyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol was synthesized according to Morita et al. (1995) with two modifications. First, Garner's aldehyde was used as a precursor to obtain the sphingosine part as described by Azuma et al. (2000). Secondly, the reaction between the sphingosine and glycosyl parts was performed according to the method described by Lemieux et al. (1975). PLGA (50:50, Resomer® RG 503H; M_w = 26,500 g/mol; M_n = 14,700 g/mol) was purchased from Boehringer Ingelheim (Ingelheim am Rhein, Germany). Fluorescent dye 1,1′-dioctadecyl 3′,3,3′,3′-tetramethylindo-carboxycyanate perchlorate (DiI), bovine serum albumin (BSA), collagenase type VIII, DNase type I, cytochalasin D, chlorpromazine and filipin III were from Sigma Chemical Co. (Saint Louis, MO). Monoclonal antibodies against mouse CD3 (APC-conjugated), B220 (PeCy7-conjugated), NK1.1 (PE- and PerCp-Cy5.5-conjugated), CD5 (APC-conjugated), CD11c (APC-conjugated), IFN-γ (PE-conjugated), IL-4 (PE-conjugated) and isotype controls were all purchased from BD Pharmingen (Le Pont de Claix, France). PE-conjugated PBS-57 glycolipid-loaded CD1d tetramer was from the NIAID Tetramer Facility (Emory University, Atlanta, GA).

2.2. Preparation of PLGA-based particles

PLGA NPs were prepared by a modified solvent diffusion (nanoprecipitation) technique (Barichello et al., 1999). Briefly, PLGA (0.9 mg) was solubilized in 80 μl acetone and 20 μl methanol. This organic phase was quickly poured, under magnetic stirring at 1000 rpm, in 1 ml deionized water (aqueous phase) containing 1 mg BSA. The nanosuspension was then stirred for 3 h to allow solvent evaporation. The loading of fluorescent probe or KRN was performed using the same protocol with slight modifications. DiI–acetone solution was added in the PLGA/acetone solution to obtain fluorescent particles. To load KRN in NPs, 100 μg KRN was dissolved in 100 μl methanol at 50 °C for 30 s. PLGA–acetone solution was added in this KRN–methanol solution and injected to the aqueous phase as described above. The produced suspensions were then kept at 4 °C. PLGA MPs were prepared by solvent evaporation method (O'Donnell and McGinity, 1996). PLGA (0.9 mg) and KRN (100 μg) were solubilized in 80 μl CH_2Cl_2 and the organic phase was dropped into the aqueous phase (1 mg BSA/ml of deionized water) under sonication for 15 s. The suspension was then stirred at 1000 rpm at room temperature for 3 h. Particles were stored at 4 °C at a final concentration of 0.9 mg PLGA/100 μg KRN/ml. To verify that DiI incorporation into PLGA-based particles was complete, NPs and MPs were centrifuged (13,000 × *g* for 30 min) and fluorescence in the supernatant was measured using a spectrofluorometer (Fluoroskan Ascent FL, Thermo Labsystems, Issy-les-Moulineaux, France).

2.3. Characterization of PLGA-based NPs and MPs

The particle size and zeta potential of PLGA-based particles were measured by a Zetasizer Nano, Malvern Instruments (Orsay, France). The particle size was examined in PBS. The surface charge was investigated in 15 mM NaCl. The morphology of PLGA-based particles was examined by transmission electron microscopy (ZEISS-EM902, West Germany) following negative staining with sodium phosphotungstate solution (0.2%, w/v).

2.4. Mice

Six to eight-week-old male wild type C57BL/6 (H-2D^b) mice were purchased from Janvier (Le Genest-St-Isle, France). The generation of CD1d$^{-/-}$ mice has been already described (Mendiratta et al., 1997). Mice were bred in our own facility in pathogen-free conditions.

2.5. Preparation of spleen and liver cells and analysis of MNC activation in response to vectorized KRN

Spleens were mechanically disrupted using a homogeneizer, washed, and red blood cells were removed with lysis buffer (Sigma–Aldrich). Livers were perfused via the venous sinus with PBS to remove circulating blood cells. Perfused livers were finely minced and enzymatically treated as described above. After washes, cell suspensions were resuspended in a 36% Percoll™ gradient, carefully layered onto 72% Percoll™ and centrifuged for 30 min at 2300 rpm, without brake at 22 °C. Mononuclear cells (MNCs) collected at the interface were washed in PBS containing 2% FCS. Spleen (1×10^6 cells/well) and liver (5×10^5 cells/well) MNCs were stimulated with increasing doses of free KRN (solubilized in 0.05% PBS/tween) or encapsulated KRN. After 48 h, the cell culture

supernatants were collected and analyzed for the IFN-γ and IL-4 concentrations using commercial ELISA kits distributed by R&D systems (Abington, UK).

2.6. Generation of BM-DCs and iNKT cell sorting

DCs were generated from the bone marrow (BM) of mice as previously described (Paget et al., 2007). In brief, BM-derived cells were cultured in RPMI 1640 medium supplemented with 10% FCS and 1% of supernatant from a GM-CSF-expressing cell line (J558-GM-CSF) (20–30 ng GM-CSF/ml). DCs were used on day 14 of culture. For iNKT cell sorting, hepatic MNCs were labelled with APC-conjugated CD5 and PE-labelled NK1.1 antibodies and cells were electronically sorted using a FACSAria (Becton Dickinson Biosciences, Rungis, France).

2.7. Analysis of iNKT activation in response to DCs previously exposed with KRN vectorized in PLGA-based particles

BM-DCs were exposed with grading doses of free or encapsulated KRN for 15 min or 2 h, washed and then co-cultured with the iNKT cell hybridoma DN32.D3 or sorted primary iNKT cells (10^5 DCs + 10^5 iNKT cells/well) in round bottom 96-well plates in RPMI supplemented with 5% FCS. After 24 h (DN32.D3) or 48 h (primary iNKT cells), IL-2, IFN-γ, and IL-4 concentrations in co-culture supernatants were measured by ELISA (R&D systems).

2.8. Analysis of PLGA KRN uptake by DCs

To assess the capacity of DCs to bind and uptake PLGA-based particles, BM-DCs (5×10^5 cells/well) were exposed with DiI-labelled particles (10 μg/ml) during 15 min or 2 h at 37 °C. After extensive washes, cells were analyzed on a FACSCalibur™ cytometer (BD biosciences) and analyzed using FlowJo software (TreeStar, Ashland, OR). The intracellular location of labelled PLGA particles was visualized under a laser scanning confocal microscope equipped with Argon (514 nm) and HeNe (633) lasers. Briefly, DCs were fixed on poly-L-lysine coated glass slides and stained with APC-labelled anti-mouse CD11c antibody. Cells were imaged with laser scanning confocal system operating on a Zeiss LSM710 microscope and a Zeiss 63× planApo 1.4 oil immersion lens. Z-stack acquisitions enabled to visualize the 3D localization of fluorescent signals. Pictures (3D reconstructions) were analyzed with Zen2008 software (Zeiss). To explore uptake pathways of PLGA-based particles, BM-DCs were pretreated for 15 min with cytochalasin D (10 μg/ml), chlorpromazine (10 μg/ml) or filipin III (1 μg/ml) prior to exposure with DiI-labelled or KRN loaded particles.

2.9. Analysis of iNKT activation, expansion and anergy in vivo

Mice were administrated intravenously with 200 μl of PBS containing or not 100 ng of free or encapsulated KRN. After 4 h, spleens were harvested and prepared as described above. Cells suspensions were incubated with appropriate dilutions of APC-conjugated CD5 and PerCP-Cy5.5-labelled NK1.1 for 30 min in PBS containing 2% FCS. Cells were then fixed in PBS 1% paraformaldehyde for 10 min, resuspended in PBS plus 2% FCS and 0.1% saponin (permeabilization buffer) and incubated for 20 min with PE-conjugated antibodies against IFN-γ, IL-4 or control rat IgG1 mAb in permeabilization buffer. Cells were acquired on a FACSCalibur™ (Becton Dickinson, Rungis, France) cytometer and analyzed using the FlowJo software (TreeStar, Ashland, OR). To quantify cytokine concentration in blood, mice were killed at various periods of time after KRN inoculation and cytokine concentrations in the sera were quantified by ELISA. To examine the iNKT expansion, blood was collected 3 days post-stimulation. Red blood cells were removed with lysis buffer (Sigma–Aldrich) and the frequency of iNKT cells was analyzed by flow cytometry using APC-conjugated CD3, PeCy7-conjugated B220 and PE-labelled PBS-57-loaded CD1d tetramer. To analyze iNKT anergy, splenocytes (1×10^6 cells/well) from KRN-injected mice were recovered 1 week post administration and stimulated with increasing doses of free KRN.

2.10. Statistics

Results are expressed as the mean ± SD or SEM. The statistical significance of differences between experimental groups was calculated by an unpaired Student's *t* test. Results with a *p* value less than 0.05 were considered significant.

3. Results

3.1. Analysis of PLGA-based particles

In this study, we generated two types of particles. As shown in Table 1, the diameter of the PLGA-based NPs, as determined by dynamic light scattering, were around 90 nm, while the MPs were approximately 715 nm in diameter. Incorporation of the fluorescent compound DiI or KRN slightly, but not significantly, modified the sizes and the zeta potentials of NPs and MPs. Analysis of polydispersity index (Table 1) and electron microscopy (Fig. 1) confirmed that NPs and MPs are both monodispersed. Quantification of DiI in the supernatants of centrifuged DiI-containing NPs and MPs revealed that the incorporation of DiI was complete (data not shown). Due to its hydrophobic nature, it is likely that the incorporation rate of KRN into PLGA-based particles is also total.

3.2. KRN encapsulated into PLGA-based particles activated spleen and liver MNCs in vitro

We next studied the biological activities of KRN encapsulated into PLGA-based NPs and MPs. To this end, PLGA particles loaded with KRN were incubated with splenocytes and, 48 h later, cytokine production was quantified by ELISA. As shown in Fig. 2A, vectorized KRN induced, in a dose-dependent manner, IFN-γ and IL-4 production by spleen MNCs. Similarly, KRN containing NPs and MPs activated liver MNCs to release cytokines (Fig. 2B). As shown in Fig. 2C, the synthesis of both IFN-γ and IL-4 was dependent on the Ag presenting molecule CD1d, thus eliminating the possibility of endotoxin contamination in the particle preparations. These data show that KRN encapsulated into PLGA can be taken up and presented by splenic and liver APC to activate, in a CD1d-dependent fashion, iNKT cells *in vitro*.

Table 1
Size and zeta potential analysis of PLGA-based NP and MP loaded or not with DiI or KRN.

Samples	Particle size		Zeta potential (mV)
	Mean diameter (nm)	Polydispersity index	
PLGA NP	90 ± 25	0.114	-30 ± 5
PLGA NP/DiI	96 ± 35	0.116	−29 ± 6
PLGA NP/KRN	105 ± 23	0.122	−27 ± 4
PLGA MP	715 ± 120	0.09	−33 ± 7
PLGA MP/DiI	702 ± 127	0.07	−27 ± 9
PLGA MP/KRN	746 ± 134	0.147	−25 ± 5

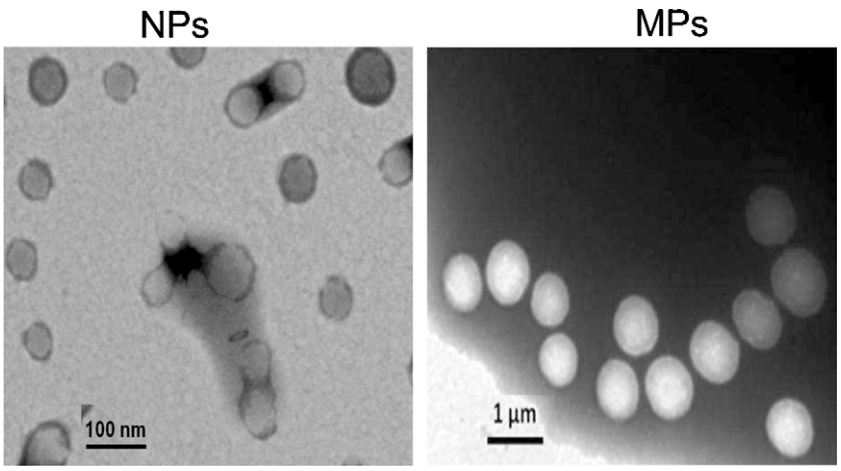

Fig. 1. Transmission electron microscopy image of PLGA-based NPs (A) and MPs (B) loaded with KRN. Particles were stained with phosphotungstic acid.

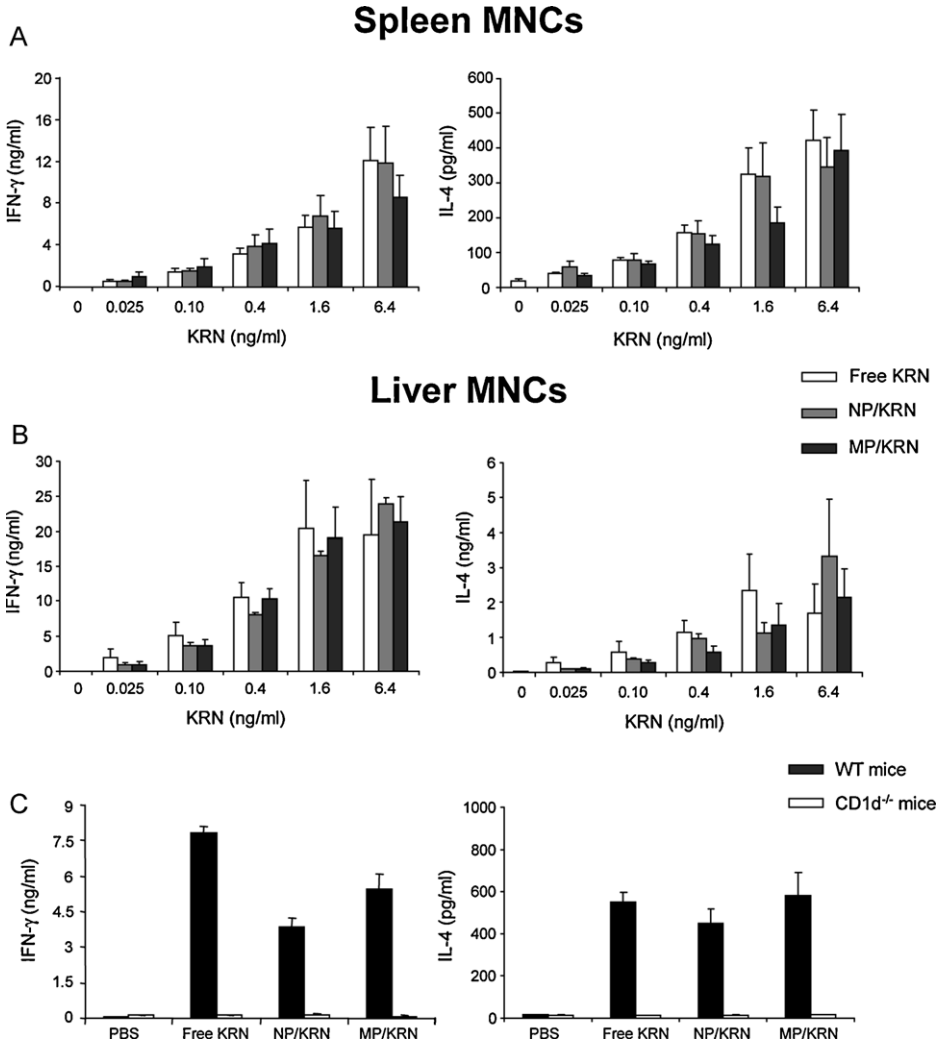

Fig. 2. Activation of spleen and liver MNCs by PLGA-based particles containing KRN. Spleen (panel A) or liver (panel B) MNCs isolated from WT mice were stimulated for 48 h with graded doses of free KRN, NP/KRN or MP/KRN. IFN-γ and IL-4 production was quantified by ELISA. (C) Splenocytes from WT or *CD1d*$^{-/-}$ animals were stimulated with free or vectorized KRN (1.5 ng/ml). Similar data were obtained when liver *CD1d*$^{-/-}$ MNCs were used (not shown). Data represent the mean ± SEM of five (panel A) and three (panel B) independent experiments performed in triplicates. One representative experiment of three performed in triplicate (±SD) is shown in panel C.

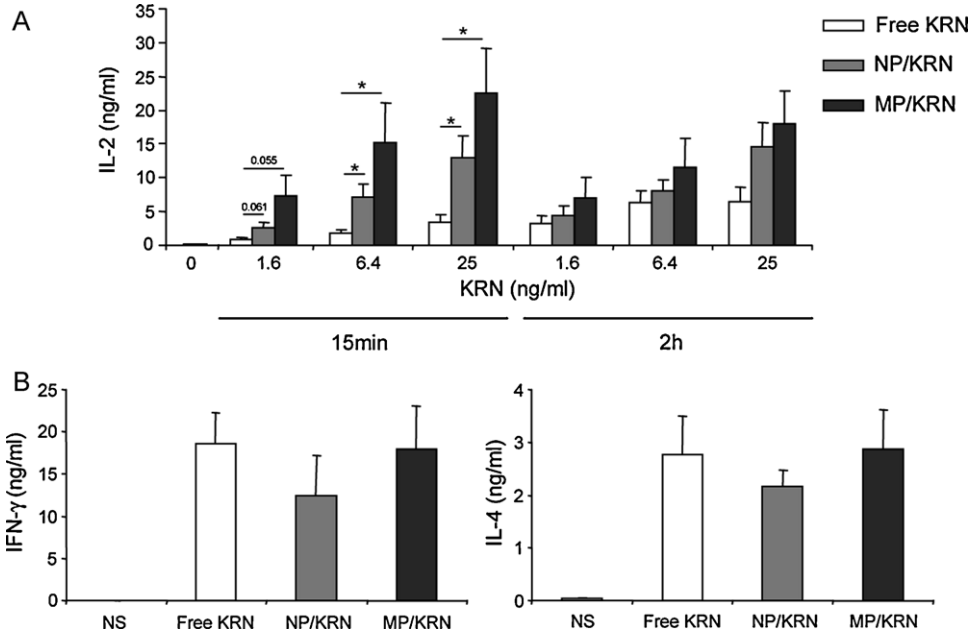

Fig. 3. Activation of iNKT cells by DCs exposed with KRN encapsulated in PLGA-based particles. (A) Various doses of free KRN or PLGA particles containing KRN were incubated with BM-DCs for 15 min or 2 h. After extensive washing, DCs were co-cultured for 24 h with the iNKT cell hybridoma DN32.D3 and IL-2 production was quantified by ELISA. Note that for the short period of sensitization (15 min), vectorized KRN induced IL-2 release from iNKT cells at a lower dose than free KRN. Of note, the particulate effect of NP/KRN and MP/KRN was also observed after several weeks of storage at 4 °C indicating a good stability of the formulations. (B) Free KRN or PLGA particles containing KRN were incubated with DCs (25 ng/ml) for 15 min and then co-cultured with sorted liver primary NKT cells ($CD5^+$ $NK1.1^+$ cells). Cytokine release was quantified 48 h later. Data represent the mean ± SEM of seven (panel A) and three (panel B) independent experiments performed in triplicate. $^*p < 0.05$.

3.3. Dendritic cells can process and present KRN formulated in PLGA-based particles to activate iNKT cells

Dendritic cells are important to activate iNKT cells *in vivo* and their targeting might be of potential interest to enhance the efficacy of KRN treatment in humans (Bezbradica et al., 2005; Chang et al., 2005; Fujii et al., 2002; Nieda et al., 2004; Parekh et al., 2005; Tatsumi et al., 2007; Toura et al., 1999). We compared the ability of KRN loaded NPs (NP/KRN) and MPs (MP/KRN) to activate iNKT cells via DCs *in vitro*. We first used the iNKT DN32.D3 hybridoma, the activation of which depending solely on CD1d/Ag mediated TCR triggering. For this, BM-DCs were incubated for various periods of time with vectorized KRN and, after extensive washes, DCs were co-cultured with the DN32.D3 hybridoma. Compared with free KRN, NP/KRN, and particularly MP/KRN, were more efficient at stimulating DN32.D3, as assessed by IL-2 production (Fig. 3A). This was significant for the short sensitization period (15 min), but not after 2 h of DC/particle contact.

We next investigated the effect of KRN vectorization on the activation of *ex vivo* sorted iNKT cells. As shown in Fig. 3B, PLGA-based particles containing KRN activated the release of both IFN-γ and IL-4 by primary iNKT cells. Unlike the iNKT cell hybridoma DN32.D3, encapsulation of KRN did not amplify the intensity of the response. Moreover, this did not modulate the IFN-γ/IL-4 ratio relative to free KRN. Collectively, DCs rapidly bind and incorporate KRN containing NPs and MPs to activate iNKT cells.

3.4. PLGA particles are endocytosed through a clathrin-dependent pathway by DCs to activate iNKT cells

Interaction and uptake of PLGA-based particles by BM-DCs was next studied by flow cytometry and confocal microscopy using DiI-conjugated PLGA particles. Sensitization of BM-DCs with DiI-conjugated NPs and MPs for 15 min, and particularly 2 h resulted in a strong labelling of DCs (Fig. 4A). At 15 min, the labelling was due to an association of the particles to the cell membrane rather than to an uptake of the particles, as revealed by confocal microscopy (Fig. 4B). In contrast, after 2 h, PLGA particles were clearly observed inside the cells as white dots whereas few fluorescence signals along the cell membrane were observed.

In an effort to identify the uptake mechanisms involved in the cellular entry of PLGA-based particles, we used several specific endocytic inhibitors. Fig. 4C shows that cytochalasin D, an inhibitor of macropinocytosis (Schneider et al., 2007) and filipin III, an inhibitor of caveolae-mediated endocytosis (Lamaze and Schmid, 1995), had no effect on the uptake of NPs and MPs. In contrast, chlorpromazine, an inhibitor of clathrin-mediated endocytosis (Wang et al., 1993), reduced by ~57% (NPs) and ~35% (MPs) the fluorescence intensities of DCs. To confirm this result, DCs were treated with chlorpromazine, just before exposure to NP/KRN and MP/KRN. Chlorpromazine, but not filipin III, reduced by ~50% (NP/KRN) and ~35% (MP/KRN) the production of IL-2 by DN32.D3 (Fig. 4D). Collectively, KRN containing PLGA-based particles are captured through a clathrin-dependent manner by DCs and this process is important to activate iNKT cells.

3.5. Inoculation of KRN formulated in PLGA activated iNKT cells and NK cells in vivo

One important property of iNKT cells is the swift release of cytokines by these cells *in vivo*. Intravenous administration of PLGA containing KRN resulted in IFN-γ production by splenic iNKT cells, as determined by intracellular flow cytometry staining (Fig. 5A, upper panel). Inoculation of NP/KRN and MP/KRN also resulted in the trans-activation of NK cells (Fig. 5A, lower panel). Thus, although no major differences with free KRN were noticed, KRN encapsulated into PLGA-based particles can activate iNKT cells *in vivo*.

Upon KRN inoculation, iNKT cells as well as other cell types activated subsequently to iNKT cell activation, release cytokines in blood. Sera were recovered 4, 16 and 24 h after the KRN treatment and then IL-4 and IFN-γ concentrations were determined by ELISA

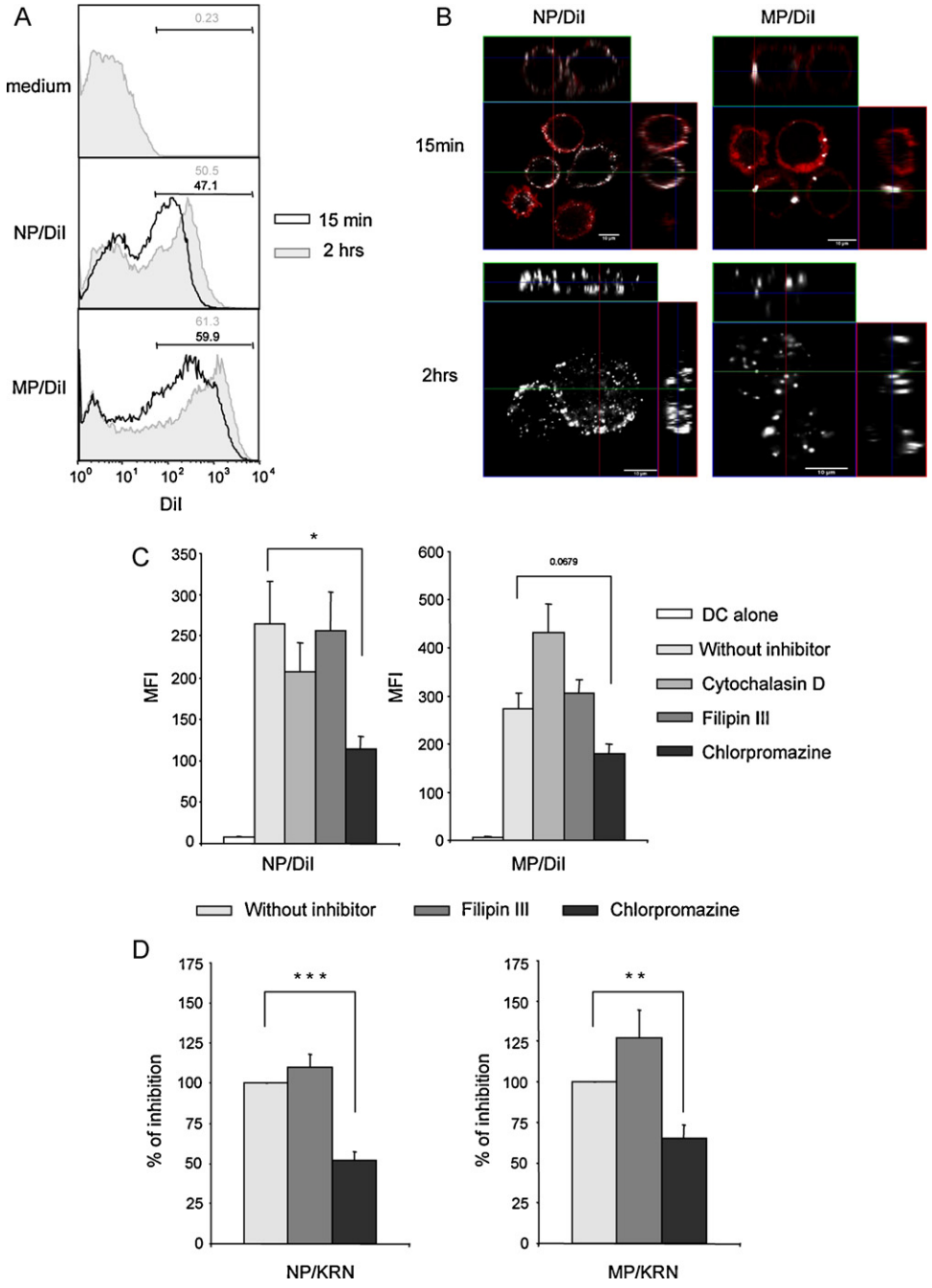

Fig. 4. Clathrin-dependent endocytosis of PLGA-based particles. (A and B) BM-DCs were exposed for 15 min or 2 h with DiI-labelled PLGA particles and then analyzed by flow cytometry (A) or by confocal microscopy (B). (A) The percentages are DiI-positive DCs are indicated for each time points. (B) Cell surface was visualized by CD11c staining (red) (upper panels). The images represent the middle focal plan (12 μm) of the DCs. Z-stack images (15–20 slices) were acquired with an interval of ~1 μm between slices. Above and on the right, views represent orthogonal cuts along the green and red lines. (C and D) DCs were pre-treated with cytochalasin D (10 μg/ml), filipin III (1 μg/ml) or chlorpromanine (10 μg/ml) and then exposed with DiI-labelled (C) or KRN containing PLGA particles (D) for 2 h. The effect of endocytic inhibitors was analyzed by flow cytometry (C) or by measuring IL-2 production by DN32.D3 hybridoma (D). (C and D) Data represent the mean ± SEM of six independent experiments performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(Fig. 5B). Incorporation of KRN into PLGA-based particles did not modify the kinetics of IL-4 (peak at 4 h) and IFN-γ release compared to free KRN. However, MP/KRN induced less IL-4 while NP/KRN had a lower capacity to promote IFN-γ release in the serum.

3.6. NP/KRN and MP/KRN differ in their capacity to expand iNKT cells and to control their recall response in vivo

Prior studies showed that, in response to free KRN, iNKT cells rapidly proliferate resulting in a significantly expanded iNKT cell population three days post-injection (Crowe et al., 2003; Wilson et al., 2003). Related to mock-treated animals, NP/KRN induced an expansion of iNKT cells in the blood although the effect was less dramatic compared to free KRN and MP/KRN (Fig. 6A).

Upon KRN stimulation, iNKT cells enter into a state of unresponsiveness that precludes further immediate restimulation (Fujii et al., 2002; Matsuda et al., 2003; Parekh et al., 2005; Singh et al., 1999; Toura et al., 1999). In agreement with other studies, and relative to naïve splenocytes, spleen cells from mice previously injected

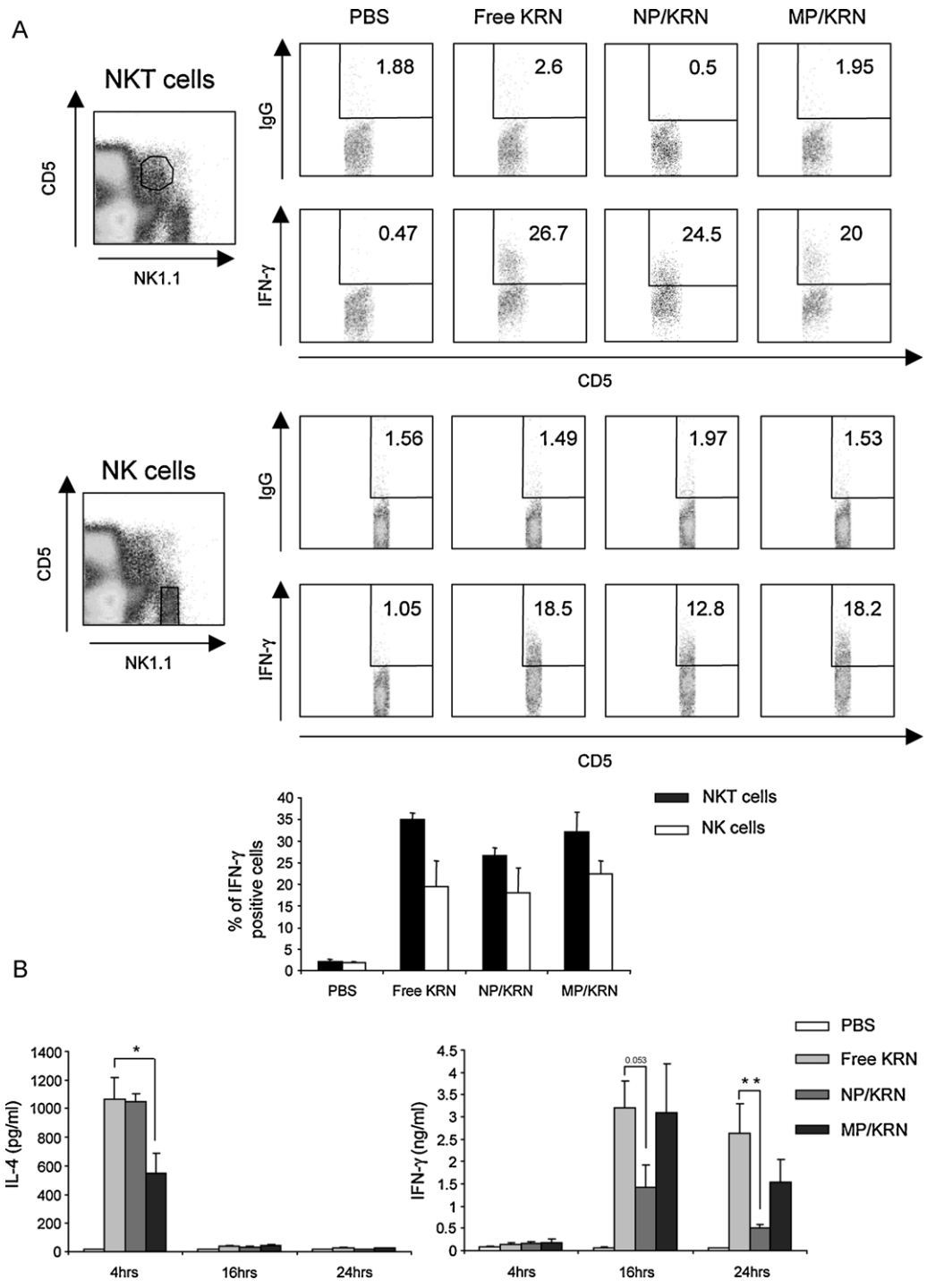

Fig. 5. Cytokine production in response to *in vivo* systemic administration of PLGA containing KRN. Mice were injected i.v. with PBS alone, free KRN or vectorized KRN (100 ng/mouse). (A) After 4 h, spleen MNCs were labelled with CD5 and NK1.1 antibodies, fixed and permeabilized for intracellular cytokine staining. Cells were analyzed by flow cytometry and gated $CD5^+$ $NK1.1^+$ (NKT) cells and $CD5^-$ $NK1.1^+$ (NK) cells were screened for intracellular IFN-γ production. Gates were set based on the isotype control. The average percentages ± SEM of NKT cells and NK cells positive for IFN-γ are represented ($n = 6$). (B) Sera were collected at different time point post-administration and cytokine concentration in the serum was determined by ELISA ($n = 6$). $^*p < 0.05$ and $^{**}p < 0.01$.

with free KRN had a lower ability to release cytokines in response to KRN re-stimulation. Similarly, inoculation of MP/KRN also resulted in a decreased response of iNKT cells upon *ex vivo* stimulation. Strikingly, the recall response of mice previously injected with NP/KRN was totally blunted. As a whole, NP/KRN and MP/KRN have a different impact on the iNKT cell behaviour following their primary stimulation *in vivo*.

4. Discussion

The synthetic α-GalCer analogue KRN7000 has a promising potential for therapeutic intervention, particularly in cancer. Many issues may however limit the clinical benefits of KRN therapy in diseases including its poor solubility in aqueous solutions, unwanted side effects (liver toxicity), the simultaneous promotion

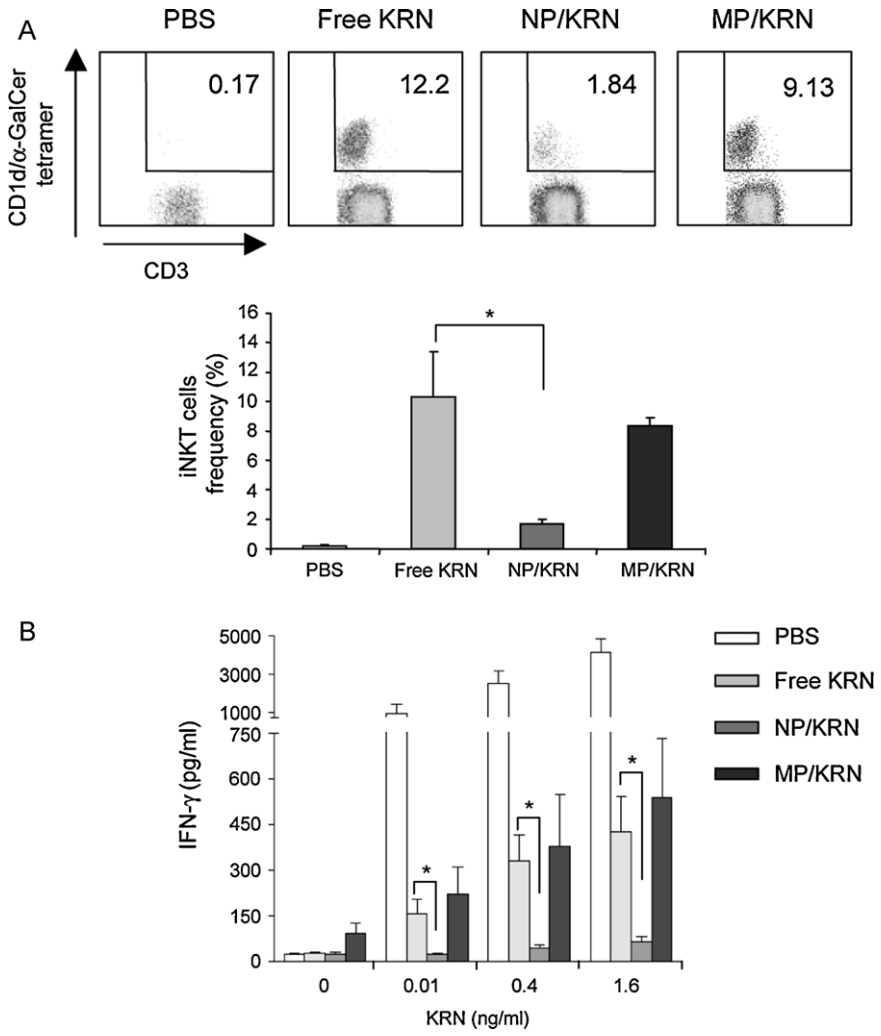

Fig. 6. Expansion and recall response of iNKT cells in mice injected with PLGA containing KRN. Mice were injected i.v. with PBS alone, free KRN or vectorized KRN (100 ng/mouse). (A) The iNKT ($B220^-$ $CD3^+$ PBS-57-loaded CD1d tetramer$^+$) cell expansion in the blood was analyzed by flow cytometry three days after injection. (B) Splenocytes were collected seven days after administration and then stimulated for 48 h with grading doses of KRN afterwards IFN-γ production was quantified by ELISA. (A and B) Data represent the mean ± SEM ($n = 6$). $^*p < 0.05$.

of a mixed Th1/Th2 cytokine response and the induction of an anergic phenotype of iNKT cells. Based on these considerations, many KRN-based analogues have been prepared to improve for instance the solubility of the active principle or to induce a more polarized immune response (Cerundolo et al., 2009; Venkataswamy and Porcelli, 2010). Surprisingly enough, very few studies have been devoted to optimize the iNKT cell response using KRN encapsulated into particles. It has been reported that α-GalCer, as well as the α-GalCer analogue threitol ceramide (ThrCer 2), formulated in liposomes promoted effective iNKT cell-mediated immune responses (Benoit et al., 2007; Kaur et al., 2011; Tamura et al., 2008). Unlike nanospheres and microspheres, liposomal formulations are unstable in nature, thus limiting their utilization. A pioneer study from Thapa and colleagues examined the effect of KRN vectorized in poly-lactic acid (PLA)-based MPs (500–1000 nm size) (Thapa et al., 2009). It was reported that this formulation is efficient at stimulating iNKT cells *in vivo*. Although PLGA has been used widely in clinical applications and is the most extensively studied polymer for encapsulating vaccine components, no attempts to incorporate iNKT cell activators into PLGA-based particles have been undertaken so far. Moreover, the effect of KRN vectorized in NPs on the iNKT cell response is presently unknown. In the current study, we used PLGA-based nano- and microparticles (90 nm and 715 nm in diameter) to characterize the iNKT cell response.

We first analyzed the potential ability of vectorized KRN to trigger cytokine production by spleen and liver MNCs *in vitro*. Our data indicated that KRN encapsulated into PLGA particles can be uptaked and presented by CD1d-competent splenic and hepatic APC to iNKT cells. In these settings, the intensity and the nature of the iNKT cell-dependent cytokine response were not different to those triggered by free KRN. This could be explained by the protocol used (48 h incubation without washes). DCs are critical to stimulate iNKT cells. Our data show that DCs rapidly bind vectorized KRN *in vitro* since 15 min exposure is sufficient to induce optimal activation of the iNKT cell hybridoma DN32.D3 (Fig. 3A). Several distinct uptake pathways may be involved in the internalization of PLGA-based particles in BM-DCs. The use of selective pharmacological inhibitors indicated that PLGA particles are internalized by DCs through a clathrin-mediated pathway. Recent evidences have shown that this mode of endocytosis is implied in delivering particulate Ag into late endosomes and lysosomes (Kitchens et al., 2006; Nam et al., 2009). Of interest, these vesicles are particularly rich in CD1d (Cohen et al., 2009). Thus, relative to free KRN, it is likely that encapsulation of KRN into PLGA particles enhanced the speed of KRN delivery into CD1d-enriched late endosomes/lysosomes, thus resulting in increased iNKT cell activation. It should be noted that for longer periods of DC contact, this effect was not observed. Activation of primary iNKT cells, unlike iNKT

hybridomas, not only depends on CD1d/Ag mediated TCR triggering but also on co-factors produced by DCs. When primary *ex vivo* purified iNKT cells were analyzed, sensitization of DCs with vectorized KRN did not enhance the amplitude of the response, unlike the DN32.D3 hybridoma. The reasons for this result are presently unknown. Moreover, as observed *in vivo* (see below), encapsulation of KRN into PLGA-based particles did not influence the polarisation (IFN-γ/IL-4 ratio) of iNKT cells.

We hypothesized that encapsulation of KRN into PLGA particles could have an impact upon the nature of the iNKT cell response *in vivo*. Our data indicate that KRN formulated into PLGA-based NPs and MPs is rapidly available to APC *in vivo* and that it activated iNKT cells with the same efficacy and kinetics as free KRN. Similarly, NP/KRN and MP/KRN induced by-stander activation of NK cells, as judged by IFN-γ production. The levels of cytokines in the serum were however different between free and vectorized KRN as MP/KRN induced less IL-4 at 4 h (probably derived from iNKT cells themselves) and NP/KRN promoted less IFN-γ release. It is likely that cells other than iNKT cells and NK cells are less activated by NP/KRN compared to free KRN or MP/KRN. One hallmark of KRN stimulation is the rapid expansion of iNKT cells. Strikingly, while administration of MP/KRN resulted in a dramatic iNKT cell expansion, we consistently observed that this phenomenon was not as potent in mice injected with NP/KRN. Our data also indicated that, contrary to our initial expectation, a single injection of vectorized KRN did not avoid iNKT cell unresponsiveness upon *in vitro* restimulation with KRN. In response to NP/KRN, iNKT cells even completely lost their capacity to produce cytokines. This phenomenon was also observed after repeated inoculation of (vectorized or free) KRN, as described in (Thapa et al., 2009) (data not shown). The mechanisms by which iNKT cells become anergized are due to the presentation of KRN by inappropriate CD1d-bearing, Ag presenting cells. Indeed, Ag recognition in the context of insufficient costimulatory signals can induce anergy. In this setting, presentation of KRN by B cells appears to promote anergy (Bezbradica et al., 2005; Schmieg et al., 2005; Toura et al., 1999) while DCs are sufficiently well equipped in co-stimulatory molecules to avoid anergy (Fujii et al., 2002; Parekh et al., 2005; Toura et al., 1999). Although our *in vitro* studies showed that PLGA-based particles can be taken up by *in vitro*-generated DCs, it is possible that, *in vivo*, they preferentially target B cells relative to DCs. In our setting, it is likely that the enhanced uptake of NPs by B cells, known to display lower endocytosis/phagocytosis activities relative to DCs, is responsible for the high level of anergy observed after NP/KRN administration. Our data do not agree with another study showing that MPs (500–1000 nm) formulated α-GalCer avoided anergy induction of iNKT cells after (repeated) systemic inoculation (Thapa et al., 2009). The reasons for this discrepancy may lie in the nature of the particles utilized (PLA *vs* PLGA) as well as the protocol used to formulate α-GalCer or KRN within the particle, which may influence Ag release and availability.

To conclude, our data validate the concept that PLGA-based particles can be used as delivery systems to activate iNKT cells *in vitro* and *in vivo*. To our knowledge, this is the first time that NP-based particles (90 nm) have been used as delivery vehicles for KRN. Owing to their superior ability to activate iNKT cells and to avoid anergy, compared with other APCs, encapsulating KRN into particulate systems that preferentially access and condition DCs *in vivo* is highly desirable. This DC-specific targeting could be based on the size of the particles and/or by the addition of specific antibodies (*i.e.* anti-CD11c) or carbohydrate ligands for DC receptors, including lectins, on the particle surface (Kwon et al., 2005; Manolova et al., 2008; Reddy et al., 2007; Tacken et al., 2007). Further improvement may also be achieved by modulating the physical stability of the particles in order to better control the KRN release kinetics into CD1d-enriched compartments. These strategies might lead to substantial clinical advantages including dose reduction, reduction of side effects and improvement of bioavailability within the targeted DCs. Because α-GalCer, and related glycolipids, have promising therapeutic activities, the development of these strategies might have important implications for the development of effective and safe iNKT cell-based immunotherapies.

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