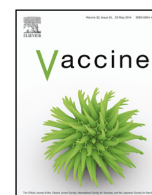
Vaccine 33 (2015) 5838–5844

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine



Synthetic TRP2 long-peptide and α-galactosylceramide formulated into cationic liposomes elicit $CD8^+$ T-cell responses and prevent tumour progression

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ARTICLE INFO

Article history:
Received 7 July 2015
Received in revised form 28 August 2015
Accepted 29 August 2015
Available online 9 September 2015

Keywords:
Cancer vaccines
Melanoma
Liposomes
Long peptides
Invariant natural killer T cells
α-Galactosylceramide

ABSTRACT

The lipid antigen α-galactosylceramide (α-GalCer) is a potent activator of invariant natural killer T-cells (iNKT cells) and can stimulate cytotoxic and anti-tumour immune responses. However optimal responses appear to be induced by α-GalCer when cell-based vaccines are delivered intravenously. Here we investigated if co-delivery of protein and peptide antigens along with α-GalCer in a liposomal formulation could stimulate therapeutic anti-tumour immune responses. Cationic liposomes were inherently immune-stimulatory and induced cytotoxic immune responses when delivered both by intravenous and subcutaneous injection. However, only vaccine delivered intravenously stimulated therapeutic anti-tumour immune responses to a peptide antigen. Surface modification with polyethylene glycol (PEG) did not improve immune responses to either intravenously or subcutaneously delivered vaccines. Immune responses to short and long peptide sequences (CD8 and CD4 epitopes) of the self-antigen tyrosinase-related protein 2 (TRP2) as a vaccine antigen, co-delivered with α-GalCer in either cationic liposomes or PBS were further examined. Enhanced production of IFN-γ, increased cytotoxic T-cell responses and tumour survival were observed when a long TRP2-peptide was delivered with α-GalCer in cationic liposomes.

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1. Introduction

Vaccination strategies have been developed to prevent tumour growth (prophylactic vaccines) or to treat existing tumours (therapeutic vaccines) by stimulating tumour-specific immunity. Prophylactic vaccination has been very successful in preventing cancers linked to viral infections, such as cervical cancer caused by the human papilloma virus or hepatocellular carcinoma caused by hepatitis B or C infections [1]. In contrast, the development of therapeutic vaccines, particularly for the treatment of melanoma, is more challenging and success thus far has been limited [2]. A number of different approaches are being investigated to create cancer vaccines that are able to induce a durable anti-tumour immune response by stimulating cytotoxic T-cell (CTL) responses [3]. These generally involve repeated immunisation with tumour associated antigens such as tumour cell lysates, purified proteins, peptides, plasmid DNA or RNA, in combination with a vaccine adjuvant or loaded *ex vivo* onto dendritic cells (DCs) [4].

Peptide vaccines offer the advantage of a good safety profile combined with ease of manufacture [3], which is important for translation into the clinic. However, the immune-stimulatory properties of therapeutic peptide vaccines need to be improved to ensure potent long-lasting immune responses. Immunisation with minimal MHC class I-binding peptides can induce immunological tolerance due exogenous loading of short peptide sequences on B- and T-cells in the absence of co-stimulatory signals necessary for the differentiation of T-cells into effector cells [5]. In contrast, immunisation with long peptides consisting of extended epitopes or the combination of several minimal epitopes requires processing by DCs prior to MHC presentation and does not result in tolerance [6].

Peptide vaccine potency can also be improved through the inclusion of danger signals, in the form of vaccine adjuvants, in the vaccine. The lipid antigen α-GalCer represents a new class of

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http://dx.doi.org/10.1016/j.vaccine.2015.08.083
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promising vaccine adjuvants that are presented on CD1d molecules on APCs and are recognised by an invariant T-cell receptor on iNKT cells. The activation of iNKT cells results in the secretion of Th1 cytokines such as IFN-γ and Th2 cytokines, such as IL-4 and IL-13 [7]. Furthermore, the reciprocal interaction of CD1d-expressing APCs and iNKT cells shapes cytokine secretion by APCs, which in turn stimulates T-cells and NK cells [8,9]. Anti-tumour immunity is primarily influenced by secretion of IFN-γ and its stimulating effect on NK cells and T-cells, which in turn recognise and lyse tumour cells [10–12]. α-GalCer has been reported to have an anti-tumour effect upon direct injection [13] and when combined with a DC vaccine [14,15]. In addition, the route of delivery of α-GalCer appears important as it has been shown that intravenous injection of α-GalCer is more potent in stimulating the expression of co-stimulatory molecules on DCs as compared to subcutaneous injection [16].

Nano-particulate delivery systems offer a possibility to circumvent laborious and expensive *ex vivo* cell therapies by simultaneously delivering antigen and α-GalCer to APCs [17–19] and by potentially being able to circumvent iNKT cell unresponsiveness, which is observed after multiple intravenous injections of soluble α-GalCer [20]. Liposomes have been thoroughly investigated for their ability to act as vehicles for drug and vaccine delivery as their amphiphilic nature allows the entrapment of hydrophilic and lipophilic drugs or antigens [21]. They also exhibit immune-stimulatory properties if they are prepared with positively charged lipids due to activation of pro-inflammatory and pro-apoptotic pathways [22] that lead to the generation of reactive oxygen species [23], enhanced expression of co-stimulatory molecules [24] and secretion of chemokines and cytokines. The addition of polyethylene glycol (PEG) to liposomal formulations has been shown to result in increased lymph node uptake of larger liposomes (>0.1 μm) and the induction of systemic responses [25,26].

Here, we were interested in investigating if the formulation of α-GalCer and antigen into liposomes could be optimised to enhance anti-tumour activity and to allow for subcutaneous administration of the vaccine. The melanocyte lineage/differentiation antigen TRP2 was used as the vaccine antigen as it is expressed by malignant melanoma cells [27].

2. Materials and methods

2.1. Preparation of liposomes containing the adjuvant α-GalCer

Liposomes were manufactured by hydrating thin lipid films [28]. For cationic liposomes 41 mg L-α-phosphatidylcholine (PC, Sigma–Aldrich, USA), 4 mg 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP, Avanti Polar Lipids, USA), 4 mg 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE, Avanti Polar Lipids, USA) and 100 μg α-GalCer (synthesised from galactose and phytosphingosine, TCI P1765, *via* α-specific glycosylation methodology [29]) were dissolved in 5 mL of chloroform and the solvent evaporated at 50 mbar at 45 °C under a nitrogen stream. A volume of 1 mL of MQ-water containing either 2 mg of the short OVA-peptide ($OVA_{323\text{-}39}$ – SIINFEKL), the long TRP2-peptide (TRP2 $_{(180\text{-}188),(88\text{-}102)}$ – SVYDFFVWLKFFHRTCKCT-GNFA), or the individual short TRP2 peptides or FITC-OVA (prepared as described previously [30]) was added, along with glass beads, to hydrate the lipid film. All peptides were purchased from Mimotopes, Australia. The formulations were placed in a water bath for 2 h at 60 °C before extrusion through stacked 800 and 400 nm polycarbonate membranes (Nucleopore®, USA) arranged in a 10 mL extruder (Lipex Biomembranes Inc., Canada).

To prepare pegylated cationic liposomes, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG (2000), Avanti Polar Lipids, USA) was added to the mixture of lipids to achieve a loading of 5% and 20%. PC liposomes were manufactured by dissolving 50 mg PC in 5 mL chloroform.

Liposomal formulations were diluted with sterile PBS prior to administration to achieve a dose of 200 ng/mouse α-GalCer, 20 μg/mouse OVA-peptide or 20 nmol/mouse TRP2 peptides.

2.2. Characterisation of formulations

Photon correlation spectroscopy was used (Malvern Zetasizer 3000, UK) to determine particle size (Z-average), polydispersity (PDI) and zeta potential of the preparations. Entrapment of FITC-OVA in liposomes was determined as described previously [31].

2.3. Co-culture of DCs and iNKTs

Aliquots of 1.25×10^5 DC224 [32] dendritic cells and 5×10^5 DN32 iNKT cells [33] (both lines provided by Ian Hermans, Malaghan Institute of Medical Research) were seeded in a 24 well-plate and incubated with either 200 ng/mL α-GalCer alone or with liposomes containing 20 μg/mL OVA and 200 ng/mL α-GalCer in IMDM at 37 °C for 24 h. An aliquot of the culture supernatants was taken and stored at −20 °C for cytokine analysis as described below. Activation of the DC224 cells was analysed by flow cytometry. Cells were stained with anti-CD11c APC and anti-MHCII FITC (BD Biosciences). The cells were washed and re-suspended in 100 μL FACS buffer containing propidium iodide. Flow Cytometry was carried out on a BD FACSCanto II and the FlowJo software (version 9.5.2, TreeStar, Inc., USA) was used to analyse data.

2.4. Cytotoxicity assay

C57BL/6 mice were bred and maintained under specific-pathogen free conditions at the HTRU, University of Otago. All experiments were approved by the Animal Ethics Committee, University of Otago and mice had free access to food and water. The mice were immunised intravenously *via* the tail or subcutaneously into the dorsal skinfold with 200 μL of the vaccine on day 0. Where indicated, two or 24 h following immunisation a blood sample was taken from the tail tip and fluorescently labelled splenocytes were injected intravenously as target cells to stimulate cytotoxic T-cell responses.

On day 7, a single cell suspension was made from the lymph node and spleen cells from naive C57BL/6 mice. Depending on the experiment, cells were pulsed with the short OVA- or TRP2-peptides (20 or 23.51 μg/mL, respectively) for 2 h at 37 °C or left untreated. Thereafter, the cells were differentially stained with CFSE (Molecular Probes, USA) as described previously [34] and injected intravenously into the tail vein of the experimental mice. The next day, mice were sacrificed and spleens were removed. The proportions of peptide pulsed cells were determined by flow cytometry BD FACSCanto II as described previously.

2.5. Cytokine assays

The collected sera were spun down to exclude cell debris from analysis and the levels of IL-2, IL-4, IFN-γ and/or IL-12p70 were measured using BD CBA Mouse Flex Sets (BD Biosciences, USA) on a BD FACSCanto II. The FCAP Array software (v1.0, Soft Flow) was used to calculate cytokine concentrations in the samples and the standards.

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