Effect of TLR ligands co-encapsulated with multiprotopic antigen in nanoliposomes targeted to human DCs via Fc receptor for cancer vaccines

Felix Ruedab, Christina Eichb, Begoña Cordobilla, Pere Domingoc, Gerardo Acostad, Fernando Albercioc, e, Luis J. Cruzf, g, Joan C. Domingoa, h

a Department of Biochemistry and Molecular Biology, University of Barcelona, Diagonal 643, 08028 Barcelona, Spain
b Department of Cell Biology Erasmus Medical Center Rotterdam, The Netherlands
c Infectious Diseases Unit, Hospital de la Santa Creu i Sant Pau, Universitat Autònoma de Barcelona, 08026 Barcelona, Spain
d Institute for Research in Biomedicine, Baldiri Reixac 10, 08028 Barcelona, Spain
e CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, 08028 Barcelona, Spain
f Experimental Molecular Imaging, Department of Radiology, Leiden University Medical Center, Leiden, The Netherlands

ARTICLE INFO
Keywords:
Delivery system
Targeting
Dendritic cells
Peptide vaccines
Immunotherapeutic vaccines
Cancer vaccines

ABSTRACT
Nanoliposomes (NLs) hold promise as new highly specific nanomedicine for anti-tumor vaccines, since they could be targeted to specific receptors on dendritic cell (DC) to induce maturation and activation and increase the anti-tumor immune response. Here we studied a NLs formulation targeted or not to FcR (the receptor for the IgG Fc fragment) for the treatment of androgen-responsive prostate cancer. Luteinizing-hormone-releasing hormone (LHRH) peptide (B- and T-cell epitopes), in tandem with a tetanus toxoid T-helper epitope (830–844 region) and several TLR (Toll-Like Receptor) ligands as adjuvants were co-encapsulated. Specific uptake in vitro of LHRH-TT liposomes targeted to the FcRs of human DCs was enhanced. DC maturation/activation, cytokine production and lymphocyte activation were consistently higher in targeted than non-targeted liposomes. Similar increase was observed as more adjuvants were administrated. Targeting to specific receptor and co-encapsulation of several TLR adjuvants are essential factors for the immune response in peptide based liposome vaccine.

1. Introduction
Tumor antigens are usually poor immunogens. However, the specificity and strength of vaccines against infectious agents (except for highly variable viruses such as HIV) and the persistence of the immune response induced has driven researchers to channel great effort into the generation of vaccines against cancer. Although the human immune system has all the machinery necessary to eradicate cancer cells and pathogens, attempts to develop effective cancer vaccines have been largely disappointing because the immune system is often inhibited in cancer patients, or tumor evasion mechanisms prevent tumor recognition (Critchley-Thorne et al., 2011; Pradere et al., 2014). Vaccination aims to induce robust and specific immune responses, but in the case of cancer vaccines it is particularly important to design strategies capable of inducing both strong cellular and humoral responses (Hutchings et al., 2005). However, three main drawbacks have impeded the rapid advance of tumor vaccines, namely, the lack of identification of tumor-associated antigens and the genetic variability of these antigens in the most common cancers; incapacity to design strategies able to overcome antigen tolerance in growing tumors (Higgins et al., 2009); and immunosuppression, associated with advanced stages of the disease, in many cancer patients. Finally, vaccination strategies disperse cancer antigens in body fluids and these molecules can be eliminated by phagocytic and reticuloendothelial uptake (reduced half-life) or they can even activate inappropriate cells. Therefore, encapsulation in liposomes or polymeric nanoparticles is a strategy to protect vaccines against degradation and target them to several receptors in dendritic cells (DCs), thus ensuring the induction of a strong and specific anti-
tumor immune response (Higgins et al., 2009). Targeting specific receptors in DCs (which act as professional antigen-presenting cells (APCs)) stimulates the maturation and activation of DCs, a cell population with an exceptional capacity to initiate both primary and secondary immune responses in vivo (Banchereau et al., 2000; Steinman, 1991). To increase the immune response, the incorporation of various adjuvants, such as Toll-Like Receptor (TLR) ligands, to vaccines, thus mimicking infection agents, could allow cancer vaccines to ultimately show a similar level of efficacy as that obtained with vaccines against infectious agents (Umar et al., 2012).

In a natural environment, there are efficient mechanisms of immunological induction against pathogens, as danger/activation signals are generated by inflammatory cytokines, which are induced by pathogens or products derived from them; nevertheless, this is not the case for the vaccines against cancer, which require strong activation, attainable only by natural or synthetic adjuvants (Guy, 2007). Several inflammatory cytokines have been used to activate DCs in vitro (namely: GM-CSF, TNF-α, IL-1β, IL-4 and IFN-γ) (Palucka et al., 2010), and some peptides derived from these cytokines or from pathogens that show adjuvant activity and could be used in vaccine formulations or conjugated with the tumor antigens on the appropriate vehicle. Pam3CysSerLys4 (Pam3CSK4) is a synthetic tripalmitoylated lipopeptide that mimics the acylated amino terminus of bacterial lipoproteins. Pam3CSK4 is a potent activator of the proinflammatory transcription factor NF-κB (Aliprantis et al., 1999). Pam3CSK4 is recognized by TLR2, present in DCs, which cooperates with TLR1 through a cytoplasmic domain to induce the activation of NF-κB through a signaling cascade (Ozinsky et al., 2000). R848 is an imidazoquinoline compound with potent anti-viral activity. This low molecular weight synthetic molecule activates immune cells via the TLR7/TLR8 MyD88-dependent signaling pathway (Henmi et al., 2002; Jurk et al., 2002). When combined with poly(dT), R848 triggers NF-κB activation in cells expressing murine TLR8 (Gorden et al., 2006). Toll-like receptor 3 (TLR3) recognizes double-stranded RNA (dsRNA), a molecular structure associated with viral infection. Polyinosinic-polycytidylic acid (poly(I:C)), a synthetic analog of dsRNA, mimics the same molecular pattern. Both natural and synthetic dsRNAs are known to induce the production of type I interferons (IFN) and other cytokines. Poly(I:C) is recognized by TLR3 (Alexopoulou et al., 2001; Matsumoto et al., 2002). Once poly(I:C) is recognized, TLR3 activates the transcription factor interferon regulatory factor 3 (IRF3), by means the adapter protein Toll-2-I receptor (TIR) domain-containing adapter, to induce IFN-β (TRIF, also known as TICAM-1) (Yamamoto et al., 2003). Activation of IRF3 is followed by the production of type I IFNs, especially IFN-β. Another pathway of this activation implicates the recruitment of TIR-related adaptor factor 6 (TRAF6) or receptor-interacting protein 1 (RIP1), with the subsequent activation of the transcription factors NF-κB and AP-1 (Kawai and Akira, 2008). Activation of this pathway triggers the production of inflammatory cytokotins and chemokines such as TNF-α, IL-6 and CXCL10. Poly(I:C) is also recognized by the cytosolic RNA helicases retinoic acid-inducible protein 1 (RIG-I) and melanoma differentiation-associate gene 5 (MDA-5) (Kato et al., 2006).

A key aspect in the development of an effective immunotherapy strategy is the selection of the targeting receptor in DCs (Tacken et al., 2011). One effective approach, based on loading DCs with antigen-IdG immune complexes (ICs), leads to efficient antigen (Ag) uptake, maturation of DCs, and increased MHC class I- and II-restricted Ag presentation in vitro (Amigorena and Bonnerot, 1999; Regnault et al., 1999; Schuurhuis et al., 2002), and T-cell priming in vivo (Akiyama et al., 2003; Kaleris and Ravetch, 2002; Schuurhuis et al., 2002). These processes are mediated by a family of FcγRs (the gamma receptors for IgG Fc fragment) consisting of the activating receptors FcγRI (CD64), FcγRIIa/c (CD16a/c) and FcγRIIIa/b (CD16a/b) and the inhibitory receptor FcγRIbb (CD32b) (Woof and Burton, 2004). Incubation of DCs with Igs activates a loss to kill signal (Bennett et al., 1998) and enables these cells to directly prime specific CD4+ (helper) and CD8+ CTL (cytotoxic T lymphocytes) responses (Bennett et al., 1998) and to induce antibody production in vivo (Getahun et al., 2004).

Here we describe an improvement of a former formulation for a vaccine to treating prostate cancer designed with a synthetic peptide that affects hormone-deprivation therapy (Cruz et al., 2011). The active components of the vaccine include a mixture of synthetic peptide Ags that direct an immune response against Luteinizing-hormone-releasing hormone (LHRH). The first development of LHRH vaccines date from the nineties. These were designed and tested in men to achieve androgen deprivation as a treatment for prostate cancer and in post-menopausal women to test the capacity to inhibit gonadotropins (Gual et al., 1997; Simms et al., 2000; Talwar et al., 1992).

The efficacy of blocking the LHRH action through the production of hormone-specific antibodies has been proved in a wide range of animal species, including humans (Naz and Rajesh, 2004). Furthermore, passive immunization has been achieved by infusion of anti-LHRH antibodies (Silversides et al., 1985), and also active vaccination with synthetic LHRH peptides coupled to tetanus or diphtheria toxoid (DT) molecules as carriers (Finstad et al., 2004; Gual et al., 1997; Simms et al., 2000; Talwar, 1997) and to multiple antigen peptide (MAP) constructs (Beekman et al., 1999).

Our strategy was to induce anti-self-immunity to LHRH by altering the target molecule on a synthetic peptide immunogen. We developed synthetic LHRH-TT (tetanus toxoid fragment) immunogens, a T helper peptide that has been used in vaccines as a clinically relevant model antigen to enhance the immune system response (Chengalvala et al., 1999; Cruz et al., 2014), and now improved it by encapsulating the antigen with several TLR ligands, compared with only one, in targeted liposomes.

2. Materials and methods

2.1. Materials and reagents

Fmoc-protected amino acids were purchased from IRIS Biotech GmbH (Marktredwitz, Germany). ChemMatrix resin was acquired to PCAs Biomatrix, (Quebec, Canada). The coupling reagents were as follows: PyAOP was obtained from Applied Biosystems (Foster City, CA), PyBOP from Novabiochem (Laufelfingen, Switzerland), HOAt from GL Biochem (Shanghai, China), and TBTU from Luxembourg Biotech. (Jerusalem, Israel). 5(6)-Carboxyfluorescein was from Acros (Somerville, NJ) and trisopropylsiline (TIS) was purchased from Fluka (Buchs, Switzerland). The other chemicals used were purchased of the highest purity commercially available. Egg phosphatidylcholine (PC), phosphatidylglycerol (PG) from egg yolk lecithin, N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP) and dithiothreitol (DTT) were from Sigma Chemical Co. (St. Louis, MO), and 1,2-dioctanoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethyleneglycol)-3400] (ammonium salt) (DSPE-PEG-Mal) was from Searlewater Polymers (Birmingham, Alabama).

2.2. Peptide synthesis and characterization

The peptide antigen X-LHRH-Ala-Ala-TTβAlaCys (EHWYSGLRPG AAQYIKANSFIGI-TELKKBAlaCys-NH2) (X = palmitic acid and 5(6)-carboxyfluorescein) was synthesized manually on aminomethyl ChemMatrix resin (0.1 mmol, 0.4 mmol/g) following standard protocols of solid-phase peptide synthesis using a Fmoc/tetr-butyl (t-Bu) strategy as described previously (Cruz et al., 2011). All the peptides were cleaved from the resin by treatment with 95% TFA, 2.5% TIS, 2.5% water for 2 h. All peptides were characterized by analytical reverse phase (RP)-HPLC (TR) and MALDI-TOF (Cruz et al., 2011).
Liposome characterization.

<table>
<thead>
<tr>
<th>Liposome Code</th>
<th>DM (nm)</th>
<th>PDI</th>
<th>Peptides</th>
<th>TLR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lip. Control (Empty)</td>
<td>125.0 ± 5.5</td>
<td>0.296 ± 0.023</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Lip. (LHRH-TT)</td>
<td>148.8 ± 10.0</td>
<td>0.358 ± 0.019</td>
<td>0.53</td>
<td>−</td>
</tr>
<tr>
<td>Lip. (LHRH-TT + R848)</td>
<td>154.4 ± 12.7</td>
<td>0.301 ± 0.027</td>
<td>0.46</td>
<td>0.26</td>
</tr>
<tr>
<td>Lip. (LHRH-TT + Poly I:C)</td>
<td>180.2 ± 10.8</td>
<td>0.336 ± 0.029</td>
<td>0.47</td>
<td>1.12</td>
</tr>
<tr>
<td>Lip. (LHRH-TT + LPS)</td>
<td>195.0 ± 15.0</td>
<td>0.409 ± 0.027</td>
<td>0.43</td>
<td>0.12</td>
</tr>
<tr>
<td>Lip. (LHRH-TT + Pam3Csk4)</td>
<td>185.6 ± 16.0</td>
<td>0.367 ± 0.028</td>
<td>0.48</td>
<td>0.26</td>
</tr>
<tr>
<td>Lip. (LHRH-TT + R848 + Poly I:C)</td>
<td>189.8 ± 19.4</td>
<td>0.380 ± 0.022</td>
<td>0.35</td>
<td>0.23 + 1.1</td>
</tr>
<tr>
<td>Lip. (LHRH-TT + R848 + Poly I:C)</td>
<td>197.6 ± 34.2</td>
<td>0.448 ± 0.028</td>
<td>0.37</td>
<td>0.27 + 1.0 + 0.12</td>
</tr>
</tbody>
</table>

Liposome Code: liposome formulation; DM: Diameter mean ± SD; PDI: polydispersity index ± SD, Peptides: mg peptides/mL liposomes and TLR: mg TLR ligands/mL liposomes. SD: standard deviation.

2.3. Preparation of liposomes, peptide encapsulation and targeted liposomes with the Fc fragment of human IgG

Unilamellar liposomes were prepared as described previously (Mercadal et al., 2000). Briefly, lipid/peptide mixtures of EPC/PG/ palmitoyl and fluorescent peptide (80/20/10 molar ratio) dissolved in methanol/methylene chloride (1:1, v/v) in a round-bottomed flask, dried and suspended in PBS buffer containing peptide (2 mg/mL) by vigorous agitation in the case of aqueous soluble peptide. Furthermore, distinct immunostimulatory TLR ligand adjuvants (R848, Poly I:C, LPS, Pam3Csk4) were added to the lipid mixture in a 10% molar ratio and the mixture subjected to 3–5 freeze-thaw cycles (liquid nitrogen-water 40 °C), followed by repetitive extrusion through Nuclepore filters (800, 400, 200 and 100 nm pore size) using a Lipex™ Extruder (Northern Lipids Inc.). Liposomes were sterilized by methanol/methylene chloride (1:1, v/v) in a round-bottomed flask.

Targeted unilamellar liposomes were produced as described above, but on this occasion a 1% molar ratio of DSPE-PEG3400-Mal was added to the lipid mixture. Thiol groups were introduced into the Fc fragments by spin column chromatography on Sepharose 4B. Analysis of peptide and TLR ligand encapsulation was performed by RP-HPLC and calculated to reach between 60 and 86% (Table 1). Vesicle size and the polydispersity index were obtained by dynamic laser light scattering using a PCS41 optic unit (Malvern Autosizer IIC).

Liposomes were characterized by transmission electron microscopy (TEM) and confocal microscopy. Liposomes were placed over on a carbon-coated Formvar carbon-coated Formvar (TEM) and confocal microscopy. Liposomes were placed on copper grids and stained with 2% uranyl acetate for 20 min. Samples observation were performed with a transmission electron microscope (JEOL JEM 1010 (Japan)) at an accelerating voltage of 80 kV. The images were obtained with a CCD Megaview III (SIS) camera (Münster, Germany).

2.4. Cell isolation and generation of human DCs

Human monocytes were purified from peripheral blood mononuclear cells (PBMCs) isolated from fresh Buffy Coats (kindly provided by the Center of Transfusion and Tissue Bank of Barcelona) by Ficoll-Paque gradient (Amersham Bioscience, Uppsala, Sweden). The interface band containing the mononuclear cells (MNCs) was withdrawn and washed several with saline; thereafter monocytes were purified by adhesion to gelatin-coated flasks. Non-adherent cells were removed and conserved frozen (10% DMSO, 90% autologous serum) for use as autologous lymphocyte source. Monocytes were differentiated to DCs as described previously (BenMohamed et al., 2002; Cruz et al., 2011).

2.5. DC maturation-activation and cytokine production

Human DCs were seeded and incubated for a period of 48 h in the presence or absence of peptide or TLR ligand encapsulated in uncoated or Fc ligand-coated liposomes. Culture supernatants were harvested and assayed for cytokine levels by means of a fluorescent immunoassay (FlowCytomix human Th1/Th2 11plex kit; Bender MedSystems GmbH). Phenotypic markers of DC maturation were analyzed by flow cytometry. Results are presented as relative expression levels by dividing mean fluorescent intensities of experimental samples by those of untreated DCs.

2.6. Uptakes of liposomes by DCs

DCs obtained as described above were incubated with labeled-peptide encapsulated in uncoated or Fc ligand-coated liposomes. Cells were incubated at 37 °C and fluorescence measures were performed at 1 h. Empty liposomes processed in the same conditions without labeled-peptides were used as negative controls. Data for at least 5 × 10⁶ DCs region/sample were acquired and analyzed on a Coulter XL flow cytometer using CellQuest and WinMDI software.

The role of FcR in the uptake of the targeted preparations was determined by means of an FcR blocking reagent (MACS, Miltenyi Biotec). Thus, when indicated, before performing the uptake assays, samples were incubated with 50 μL FcR blocking reagent (MACS, Miltenyi Biotec) for each 1 × 10⁶ DCs for 30 min at 4 °C, after which the tests were carried out in the same conditions as described above.

Confocal microscopy was used to confirm internalization of labeled-peptide encapsulated in liposomes. DCs attached to poly-L-lysine coated glass slides were stained with anti-human MHC class II antibody (clone Q5/13) or IgG2a isotype control, followed by a secondary mAb goat-anti-mouse Alexa 647 antibody. Cell images were obtained with a Bio-Rad MRC 1024 confocal system running on a Nikon Optiphot microscope and a Nikon 60 × planApo 1.4 oil immersion lens. Pictures
2.7. DC stimulation of autologous lymphocytes

Primary DCs were loaded with liposomes as described above and treated for 30 min with Mitomycin C (50 μg/mL final concentration) to prevent proliferative activity, then washed two times with saline and co-incubated with lymphocytes. Autologous stimulation was conducted in 96-well culture plates in 200 μL of RPMI 1640 medium containing 10% of inactivated FCS (fetal calf serum) and antibiotics (BenMohamed et al., 2002; Cruz et al., 2011). T cell proliferative responses were studied indirectly by the radioactivity incorporation into DNA after 18 h. Measure of radioactivity was performed in a beta counter (Beckman 6500 LS) in 5 mL of scintillation liquid (Opti-Phase HiSafe, PerkinElmer). Results are expressed as cpm.

2.8. Measure of cytokine production on T lymphocytes

Culture supernatants from the stimulated T lymphocytes were harvested at 72 h of culture and frozen at ~80 °C until use. Measure of cytokine production was carried out by fluorescent bead immunoassay (FlowCytomix human Th1/Th2 11plex kit; Bender MedSystems GmbH).

3. Results

3.1. Preparation and characterization of targeted liposomes encapsulating TLR ligands and peptides

The chimeric immunogens, containing both the decapeptide targeted to LHRH site and a helper T-cell epitope, were produced by a solid-phase approach. The two domains (LHRH and T epitope) were separated by two Ala residues as spacer. Two N-terminal modifications of peptide were performed as follow: i) carboxyfluorescein, to allow tracking by flow cytometry; and ii) palmitoylation, to improve the encapsulation in liposomes. These modified peptides were characterized by RP-HPLC and mass spectroscopy (as described in materials and methods).

The liposomes designed as showed in the general diagram of Fig. 1B, were analyzed by mean diameter, polydispersity index (PDI), and encapsulation efficiency. As it is shown in Table 1, the sizes of liposomes varied from 125.0 ± 5.5 to 207.8 ± 36.7 nm, for the empty ones to those more complex (Fc targeted and encapsulating LHRH-T-T + R848 + Poly I:C + LPS), respectively, which were desirable for vaccine formulations. Liposomes prepared using all the methods were also homogeneous and had a uniform size with monomodal distribution (Table 1). Co-encapsulation of different peptides resulted in entrapment efficiencies of the peptides similar for the different preparations performed (Table 1). Co-encapsulation of different peptides resulted in entrapment percentages similar to monocomponent systems.

3.2. Effect of targeting on the uptake of liposomes by DCs

Liposomes loaded with fluorescent-labeled peptides and targeted or non-targeted to the FcγR (the receptor of the constant fraction of IgG) were incubated with DCs at 37 °C (Fig. 2). The presence of the Fc fragment from IgG on the surface of the liposomes induced a significant effect on the uptake by human DCs. Cells were analyzed by confocal laser scanning microscopy. Cell surface was visualized by MHC class II staining (blue). The image represents the middle focal plane of the DCs.

Fig. 2. Uptake of liposomes by DCs.
A) Liposome uptake was examined in DCs collected and incubated with FITC-labeled-peptide encapsulated in liposomes with or without attachment of the Fc fraction of human IgG (black bars). Cells were incubated at 37 °C, and fluorescence measures were performed at 1 h. DCs alone processed in the same conditions were used as negative controls. Data for at least 5 × 10^3 DCs region/sample were acquired and analyzed on a Coulter XL Flow cytometer using CellQuest and WinMDI software.
B) DCs were incubated with FITC-labeled-peptide encapsulated in liposomes (green) for 1 h to confirm uptake by human DCs. Cells were analyzed by confocal laser scanning microscopy. Cell surface was visualized by MHC class II staining (blue). The image represents the middle focal plane of the DCs.

Fig. 1. Characterization and schematic diagram of targeted liposomes to human DCs via the FcR.
A) TEM images of liposomes, after staining with 2% uranyl acetate, the dark color is due to the interaction between lipids from the liposomes and uranyl acetate; B) schematic diagram of targeted liposomes to human DCs via the FcR. Distinct targeting and non-targeting liposomes carrying MHC class II and class I-restricted LHRH peptide antigen and/or in combination with adjuvant peptides were generated. Targeted liposomes were prepared with a lipid-PEG layer to which the Fc fragment from IgG on the surface of the liposomes induced a significant

Fig. 1B) DCs were incubated with FITC-labeled-peptide encapsulated in liposomes (green) for 1 h to confirm uptake by human DCs. Cells were analyzed by confocal laser scanning microscopy. Cell surface was visualized by MHC class II staining (blue). The image represents the middle focal plane of the DCs.
increase in the uptake of the targeted liposomes with respect to non-targeted ones (Fig. 2). The binding specificity of the Fc targeted liposomes to the FcR on DCs was demonstrated since only the uptake of the liposomes carrying the Fc fragment was inhibited when the blocking agent was added (Fig. 2).

3.3. Induction of activation/maturation and cytokine production by DCs primed with liposomes loaded with TLR ligands and peptide tumor-antigen targeted or non-targeted to Fcγ receptor

Human DCs were primed in vitro with several liposomal preparations, targeted or not to the Fcγ receptor, containing a LHRH-TT peptide and one, two or three adjuvants. Several phenotype markers of DCs, related to activation/maturation and antigen presentation, were used to establish the influence of the different components in the liposomal formulations on DC functionality. In general, all the Fc-targeted liposomal preparations were superior to their non-targeted counterparts for all the markers and cytokines studied (Fig. 3). However, when liposomes did not contain any adjuvant, no differences were observed between targeted and non-targeted liposomes. The most striking effect observed on activation/maturation of DCs was in presence of the TLR ligand 4, LPS, and when two or three adjuvant molecules were co-encapsulated (Fig. 3). The effect of FcR-targeted liposomes containing Poly (IC), which interact with TLR3, on maturation/activation markers is also worthy to be mentioned (Fig. 3). When production of cytokines from iDCs loaded with the above mentioned targeted and non-targeted liposomal preparations were studied, the cytokine patterns (e.g. IFN-γ, TNF-α, IL-6 and IL-8) were very similar to those described for the phenotypic maturation/activation markers. Only the cytokines presented yield measurable values among those studied (Fig. 4).

3.4. Induction of autologous lymphocyte proliferation and cytokine production by DCs loaded with multiple TLR ligands and peptide encapsulated in liposomes

Lymphocyte proliferation was studied by co-culture of autologous lymphocytes with DCs primed with distinct targeted and non-targeted liposomal formulations with two concentrations (125 and 250 ng/mL) of encapsulated peptides plus various combinations of adjuvants.

As expected, it was observed a parallelism between the results of the lymphocyte proliferation and those found in the DC activation phenotype after priming with the targeted and non-targeted liposomes (Fig. 5). That is, under identical conditions, the targeted liposomes were consistently higher than non-targeted ones in the proliferative response induced. Nevertheless, the relevance of the adjuvants in the lymphocyte response was evidenced by the higher induction of non-targeted liposomes containing three distinct immunostimulatory TLR ligands adjuvants (namely: R848, Poly I:C, LPS) than the targeted liposomes containing only one adjuvant molecule (Fig. 5). In all cases, an antigen concentration of 250 ng/mL induced a higher response than liposomes.
loaded with 125 ng/mL, thereby suggesting that the adjustment of antigen concentration is critical to attain an optimal response.

Supernatants from lymphocytes stimulated with DCs primed with different formulations of targeted and non-targeted liposomes were collected, and the cytokine content was studied. The pattern of cytokine production was very similar to that observed for lymphocyte proliferation. Targeted liposomes gave higher cytokine production than untargeted and co-encapsulation of three TLR ligand adjuvants induced higher cytokine production than two or one (Fig. 6). Among the cytokines studied, IL-8 and IL-4 were produced in greater amounts, followed by IL-2, IFN-γ, TNF-α and IL-6 which were also produced in appreciable concentrations.

4. Discussion

It is undeniable that vaccination has changed the history of humanity about their biggest challenge in the past, infectious diseases. The new challenge is to master the capabilities of our immunological system to fight against cancer. During the last decades our understanding of tumor biology and the immune system have achieved promising immunotherapies for melanoma and other cancers such as lung and kidney. New agents for the treatment of metastatic cancer based on their strength to enhance patients’ immunological response have been approved by drug regulatory agencies and world health organizations. Likewise, treatments to control checkpoint inhibitors of the immune system.
system that tumors use to evade T cell antitumor response are currently being investigated.

A successful strategy for cancer vaccines has been hindered by several limitations. Firstly, tumors show high genetic variability, which leads to loss of antigen targets; therefore, a new vaccine should include multiple antigens to prevent tumors evading immune recognition. Secondly, tumor antigens are close to their normal protein counterparts (often point mutations or newly expressed fetal antigens). Therefore, immunologic therapies against cancer must trick the immune system into believing that it is facing infectious agents, thus inducing a strong immune response (e.g. target tumor antigens to TLRs). Thirdly, cancer antigens injected non-encapsulated are dispersed in body fluids (with reduced half-life) and could even activate inappropriate cells. Therefore, in order to preserve vaccines against degradation and to ensure targeting of several DC receptors, which are specialized in antigen presentation, stimulate DC maturation and activation, and enhance the induction of anti-tumor responses, they should be encapsulated in polymeric or liposomal nanoparticles of an appropriate size to reach the target tissues. If all these traits are taken into account then it would be possible to develop a vaccine that meets expectations and that yields results similar to those obtained with vaccines against infectious agents (Umar et al., 2012).

DCs are key regulators of the immunological system. They take up, process, and present substances (antigens) recognized by the adaptive immune system (T- and B-lymphocytes) and initiate an immune response. Given their properties, DCs have been used in cell-based antitumor vaccines, some of which are currently in clinical trials. However, although these trials have corroborated proof of principle and have shown that the efficacy of the vaccine improves over time (Cintolo et al., 2012), clinical efficacy has been limited, and many aspects of tumor immunology and basic DC biology remain unknown. These limitations and the technically complex in vitro development of DCs have prevented global optimization and application of these DC-based vaccines (van Vliet et al., 2007).

To date, most nanovaccines that target DCs rely on antigens which are chemically linked to an antibody that recognizes these cells. However, as with the activation of potent T cell responses, the activation and maturation of DCs and the attainment of a migratory phenotype by this cell type require proper immune signals. Recent studies suggest that optimal antigen presentation requires that antigens and DC activation stimuli (namely TLR ligands) be located in the same intracellular compartment (Delamarre et al., 2006). Furthermore, free adjuvants are less efficient stimulating immunological response that the encapsulated one (data not shown) (Cruz et al., 2011). Additionally, unlike tumor antigens, pathogen antigens as TLR ligands induce a strong immune responses through specialized receptors on DCs.

Our liposomal nanovaccine contains all the components required to generate the signals necessary to trigger an effective immune response (Fig. 1B): 1) targeting of activation/maturation receptors on DCs via Fc Receptor; 2) pathogen recognition receptors (PRR) and activation...
signals, by means of TLR ligands; and 3) tumor antigens covering a broad spectrum of tumors with epitopes for HLA classes I and II (Rammensee et al., 1999).

We studied distinct liposomal nanoformulations for the development of a vaccine for the treatment of androgen-responsive prostate cancer comparing targeted and not targeted to FcR (the receptor for the IgG Fc fragment). To this end, palmitoylated immunogenic peptide containing the LHRH peptide (B- and T-cell epitope), in tandem with a T-helper epitope corresponding to the 830–844 region of tetanus toxin was encapsulated. In addition, several TLR (Toll-Like Receptor) ligands (R848, Poly I:C, LPS, Pam3CSk4) were co-encapsulated as adjuvants. Intracellular delivery of LPS initiates only TRIF-dependent signaling via clathrin-mediated endocytosis, which is independent of CD14[44]. This TLR-dependent pathway, by activation of IRF-3 and NFkB, induces the expression of type-I IFN, and RANTES, which are important for the activation of adaptive immune responses. As demonstrate by Watanabe S et al. (Watanabe et al., 2013), LPS encapsulated in liposomes strongly activate a pathway that is a TRIF-dependent signaling without inducing unnecessary inflammation. The additive or synergistic activity of these TLR ligands was assayed. In this strategy, DCs are activated and the uptake of targeted and non-targeted molecules, Uptake of targeted liposomal systems containing tumor antigen co-encapsulated, is important for DCs to break and avoid the induction of tolerance (Bosccardi et al., 2006).

Our results have shown that liposomes containing LHRH-TT targeted to the FCRs of human DCs induced a strong immune response with respect to non-targeted molecules. Uptake in vitro was enhanced in the targeted liposomes and was partially specific as demonstrated by the drop in liposome incorporation observed after using an FCR blocking reagent, which confirm something known; this is that DCs present different pathways of phagocytic activity beside those of the specific receptors. Targeted liposomes showed a higher response with respect to non-targeted in DC maturation/activation markers, cytokine production and lymphocyte activation. Despite that Fc-gammaRs mediate internalization of IgG complexes can promote DCs maturation (Amigorena and Bonnerot, 1999; Regnault et al., 1999), targeted empty liposomes did not show any effect on DC maturation, possibly the absence the antigen and the number of Fc incorporated in each particle could explain this lack of apparent stimulation.

As previously presented (Chengalvala et al., 1999), this in vitro immunization system allows rapid and even high-throughput screening of distinct strategies for immunotherapeutic development. Once again it has been demonstrated, that target activating DC receptors, such as FcR or other danger receptors, is one of the best methods to transport antigens to APCs for vaccination. Our results demonstrate the feasibility of targeted liposomal systems containing tumor antigen co-encapsulated with several adjuvants and the importance of incorporate more than one adjuvant activating different pathways to induce a potent immune response in the development of a novel strategy for cancer vaccines.

5. Conclusions

One of the purposes of this paper is the observation that co-encapsulation of the three TLR ligands acting in parallel, which use not identical activation pathways, have an additive or synergic role, yielding the most striking differences of the liposomal formulation, showing the importance of this kind of adjuvant in the immune response. The other goal of this work is to show the utility and efficiency of formulate a vaccine with all the components in side of a liposome targeted to a DC-activating receptor as Fc-Receptor.

Conflict of interests

None declared.

Acknowledgements

This work was supported by GICYT (CTQ2009-07758, CTQ2008-00177), the Instituto de Salud Carlos III (CB06_01_0074), by a Grant from the IV Convocatoria de Ayudas a la Investigación de la Fundación Mutua Madrileña and the Generalitat de Catalunya (2009SGR 1024 and 2009SGR 367).

References


