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Immunogenicity of lipid sustained release implants containing imiquimod, α-galactosylceramide, or Quil-A

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The aim of this study was to investigate and compare the immunogenicity of liposome-forming, sustained release lipid implants containing either an α-galactosylceramide (α-GalCer) analogue, imiquimod or Quil-A (QA) as adjuvants. Ovalbumin (OVA) was used as a model antigen. Groups of C57Bl/6 mice were subcutaneously immunised with lipid implants containing one of the adjuvants, or inoculated with OVA in alum. The expansion of $CD8^+$ and $CD4^+$ transgenic T cells was analysed to assess the ability of these implants to stimulate cell-mediated immunity. In addition, the production of OVA-specific IgG antibodies was determined. QA-containing lipid implants were more efficient in the stimulation of $CD8^+$ T cells and IgG antibodies than the two immunomodulators α-GalCer and imiquimod. These results suggest that, using this immunisation protocol and dose of immunomodulators, QA was superior to imiquimod and α-GalCer.

1. Introduction

The development of controlled or sustained release formulations for the delivery of drugs and vaccines is a growing trend. Lipid implants have been investigated since the 1990s, with implants being prepared predominantly from cholesterol or phospholipid-cholesterol mixtures (Demana et al. 2005; Khan et al. 1991, 1993; Walduck et al. 1998). More recently triglycerides and blends of triglycerides with phospholipids and cholesterol have been used (Guse et al. 2006a, b; Herrmann et al. 2007; Koennings et al. 2007; Mohl and Winter 2004; Vogelhuber et al. 2003). The advantages of lipid implants for use as controlled or sustained release formulations are the good safety profile, biocompatibility and biodegradability of most lipids, and ease of manufacture of most lipid-based delivery systems. The prolonged release of an optimised dose of vaccine antigens could potentially enhance uptake of the antigen by antigen presenting cells due to a longer interaction between cells of the immune system and the antigen (Zinkernagel et al. 1997). The sustained delivery of the antigen in an immunogenic manner should also lead to a reduced need for booster injections. However, in order to avoid the development of immune tolerance, adjuvants must be incorporated into the formulation and antigen must be released in an immunogenic form. Particulate carriers have been shown to induce stronger immune responses than the delivery of soluble antigen, as particles can be regarded as dangerous by the immune system of the host (Burstein et al. 1992). In addition, particulate carriers can potentially mediate the delivery of antigen and adjuvant simultaneously to a cell of the immune system (Bramwell and Perrie 2005).

We were recently able to demonstrate that liposome-forming sustained release lipid implants containing the adjuvant QA were able to stimulate immune responses comparable to those produced in response to two doses of an immediate release formulation (Myschik et al., J. Drug Target., accepted for publication). QA is a triterpene saponin adjuvant extracted from the bark of the soapbark tree (*Quillaja saponaria* Molina). The *Quillaja* saponin triterpenes differ in their sugar side chains and adjuvant activity (Barr et al. 1998). Some QA fractions have been shown to stimulate Th1-type immunity and are able to activate cytotoxic T lymphocytes (Behboudi et al. 1999; Johansson and Lövgren-Bengtsson 1999). QA also exhibits haemolytic activity due to its surfactant nature when administered in high concentrations, a disadvantage that currently limits the use of QA in vaccines for humans (Kensil 1996). This drawback can be partially overcome by the incorporation of QA into a particulate carrier, such as liposomes or immunostimulating complexes (ISCOMs) (Kersten et al. 1991; Rönnberg et al. 1995). An additional issue with the use of QA in human vaccines is the mixture of different saponins in the extracts and that therefore they do not consist of a single, defined chemical entity (Kensil et al. 1991). Attempts to clarify which QA fraction is responsible for the immune activation have been undertaken and a number of saponins with immune stimulating activity were identified (Johansson and Lövgren-Bengtsson 1999; Soltysik et al. 1995) of which one is shown in Fig. 1. The aim of this study was to determine if other adjuvants can be incorporated into the lipid implants and evoke cellular and humoral immune responses equivalent or better than those induced by QA. Two chemically defined synthetic immunomodulators, an α-galactosylceramide (α-GalCer) analogue and imiquimod, which have well defined mechanism of action (Hayakawa et al. 2004; Marciani 2003; Schiller et al. 2006; Stanley 2002), were investigated.

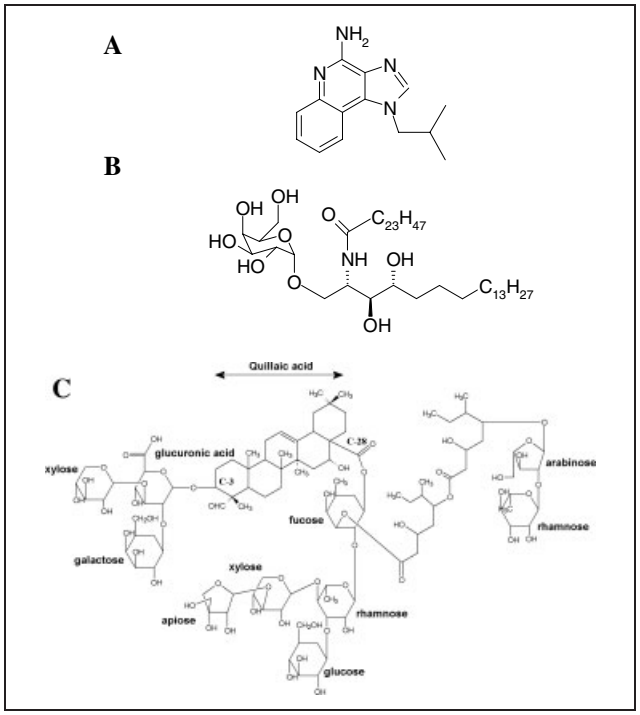

Fig. 1: Chemical structures of **(A)** Imiquimod, **(B)** α-GalCer analogue and **(C)** Quil-A (showing only QS-21, (Lendemans 2006) taken from Soltysik 1995). The triterpene saponin backbone and the sugar residues are indicated

Imiquimod is a small molecular weight compound belonging to the family of imidazoquinoline compounds, which have been termed immune response modifiers or IRMs. Imiquimod has been reported to modify innate immune responses and stimulate cell-mediated immunity and the development of Th1 immune responses (Wagner et al. 1999). Cell-mediated immunity is desirable for protection against or the treatment of cancer or infections that originate from intracellular pathogens. Imidazoquinolines act as agonists on endosomal Toll-like receptors (TLRs) (Akira 2003). Imiquimod binds to TLR7 and 8 in humans, and TLR7 in mice, which are TLR8 deficient (Weeratna et al. 2005). Following the engagement of imiquimod with the TLRs, the MyD88 cascade is activated (Hemmi et al. 2002; Schiller et al. 2006), leading to the production of a specific pattern of cytokines, including interleukin (IL)-1, IL-6, IL-12, interferon-α, interferon-γ, and TNF-α (Wagner et al. 1999). Thus, imiquimod stimulates the production of endogenous cytokines, and exhibits indirect antiviral activity. In addition, drugs of the imidazoquinoline family in general have been reported to activate B cells and, in turn, the production of antibodies (Tomai et al. 2000). Imiquimod has been typically used as a topical IRM, and is usually applied to the skin in the form of a 5% cream. Imiquimod has been approved by the FDA for the topical treatment of warts, actinic keratosis, and superficial basal cell carcinoma (Gupta et al. 2004). The subcutaneous administration of imiquimod that is the focus of our study has been less frequently investigated and in addition, the administration of imiquimod in a slow release formulation has, to the best of our knowledge, not been reported. Nonetheless, Weeratna et al. (2005) suggested that a formulation which is able to provide a depot might enhance the adjuvant effect of small immune response modifiers (IRM) such as imiquimod, as IRMs are likely to have a different biodistribution compared to larger adjuvant molecules due to their small molecular size.

α-GalCer is a glycolipid which was first discovered in extracts from the marine sponge *Agelas mauritianus* (Natori et al. 1994). Its adjuvant activity occurs through binding of α-GalCer to the non-polymorphic protein CD1d that is expressed on antigen-presenting cells (Naidenko et al. 2000; Tsuji 2006). Binding of α-GalCer leads to the activation of invariant NKT (*i*NKT) and the production of interferon-γ and IL-4. α-GalCer has been shown to be a potent stimulator of cellular immune responses and has been used in clinical trials (Giaccone et al. 2002; Ishikawa et al. 2005). The α-anomeric conformation of the sugar-ceramide moiety is a chemical orientation that has been found in bacteria but does not occur in mammals, emphasising that α-GalCer is probably not a natural ligand for CD1d (Lawton and Kronenberg 2004). The α-orientation appears to be essential for the receptor binding and adjuvant activity (Borg et al. 2007). Molecules bearing the β-conformation had first been reported to feature little or no activity when compared to α-GalCer (Kawano et al. 1997) but have more recently been proposed to simply express an activation mechanism different from that of α-GalCer (Brutkiewicz 2006; Parekh et al. 2004). Like imiquimod, α-GalCer is produced by chemical synthesis (Lee et al. 2006), leading to one defined chemical entity (Fig. 1). The α-GalCer analogue utilised in this study differs from the original compound α-GalCer (KRN7000) in the lengths of the lipid acyl chain and the sphingosine moiety by 2 C-atoms, respectively. Previous studies have shown that this analogue has the capacity to stimulate *i*NKT cells *in vivo* with less potency than α-GalCer but also reduced the Th2 bias reportedly stimulated by the truncated α-GalCer analogue OCH (Lee et al. 2006).

2. Investigation and results

2.1. Release of particles from adjuvant containing implants

All implants released liposomes upon hydration with buffer as depicted in Fig. 2. The liposomes were predominantly multilamellar and varied in size. Sustained release of antigen from the implants was achieved by the addition of excess cholesterol to the implants as described elsewhere (Myschik et al. J. Drug Target., accepted for publication), which resulted in a release of antigen over a period of 10 days *in vitro*.

2.2. Expansion of transgenic CD8$^+$ T cells in vivo after vaccination with implants containing α-GalCer, imiquimod or QA as adjuvants

The ability of the implant vaccine formulations to stimulate an expansion of CD8$^+$ T cells *in vivo* was investigated. CD8$^+$ T cells have the ability to convert into cytotoxic T lymphocytes (CTL) which can then kill pathogen-infected cells or tumours. The initiation of a CD8$^+$ T cell response as part of cellular immunity is crucial, and inherently more difficult to achieve by a vaccine than are humoral immune responses.

After adoptive transfer of OT-I and OT-II transgenic T cells, which provide a tracer population of T cells that can be easily and specifically analysed by flow cytometry, mice were immunised as described in the Table. The OVA implant group was included as a negative control as pre-

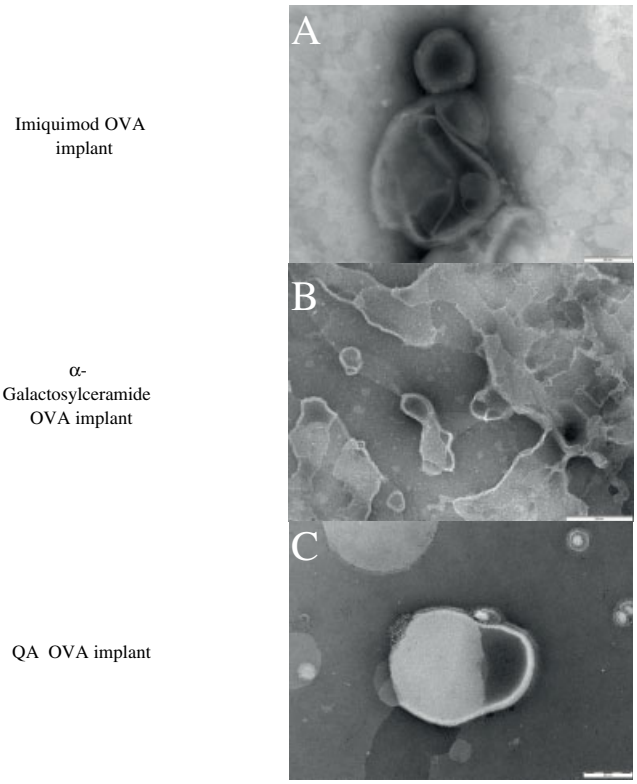

Fig. 2: Transmission electron micrographs of liposomes released from lipid implants upon hydration with buffer. **(A)** Imiquimod OVA, **(B)** α-GalCer OVA and **(C)** QA OVA implants were placed into 400 µl of PBS buffer pH 7.4 at 37 °C. Samples of 50 µl were withdrawn of which 10 µl were then placed onto a glow-discharged carbon-coated copper grid and subsequently stained with 2% phosphotungstic acid pH 5.2. Bar = 200 nm

Table: Immunisation protocol and composition of the different formulations used for the assessment of the quality of immune response evoked towards the formulations

Group	Formulation	Adjuvant	Amount of antigen	Immunisation time points
A	Imiquimod OVA implant	Yes	20 µg OVA	Day 0
B	α-GalCer OVA implant	Yes	20 µg OVA	Day 0
C	QA OVA implant	Yes	20 µg OVA	Day 0
D	OVA implant	No	20 µg OVA	Day 0
E	alum + OVA	Yes	10 µg OVA	Day 0, 14

vious work (Myschik, et al. 2008b) has shown this immunisation regime stimulated immune responses similar to those induced by OVA in PBS. All mice received a boost of soluble antigen in PBS on day 29 and were sacrificed three days later. Axial and brachial lymph nodes, spleens and blood were collected.

The analysis of transgenic $CD8^+$ T cell expansion in the cell suspensions using flow cytometry revealed that only the QA OVA implant achieved a significantly larger expansion of $CD8^+$ T cells in both the draining axial and brachial lymph nodes as compared to the OVA implant and OVA in alum (Fig. 3). All implants, including the OVA implant which did not contain an adjuvant were as effective in stimulating the expansion of transgenic $CD8^+$ T cells as were two immunisations of OVA in alum.

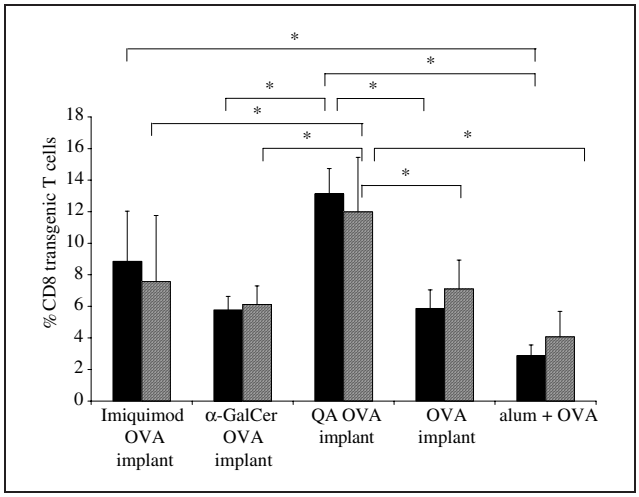

Fig. 3: Expansion of transgenic $CD8^+$ T cells, as a percentage of total CD8 T cells, in lymph nodes and spleens obtained from C57Bl/6 mice on day 33 of the study. Black bars represent the results obtained from splenocytes; dashed bars represent the results obtained from lymphocytes. Data was pooled from three independent experiments (n = 2–3 mice in each experiment) and is expressed as mean ± S.D. Statistical differences were determined using ANOVA followed by Tukey's pairwise comparison with $p \leq 0.05$ regarded as significantly different. Significant results are indicated by an*

2.3. Expansion of T cells recognising the CD8 epitope of OVA (SIINFEKL) in vivo after vaccination with implants containing α-GalCer, imiquimod or QA as adjuvants

We next investigated the expansion of T cells recognising the CD8 epitope of OVA (SIINFEKL) using a SIINFEKL-specific pentamer. This method allows for the detection of all $CD8^+$ T cells recognising SIINFEKL, not only the transgenic T cells. The trend of expansion of $CD8^+$ T cells was very similar to that observed for the transgenic T cells. Again, the QA OVA implant induced the strongest expansion of $CD8^+$ T cells. This difference was signifi-

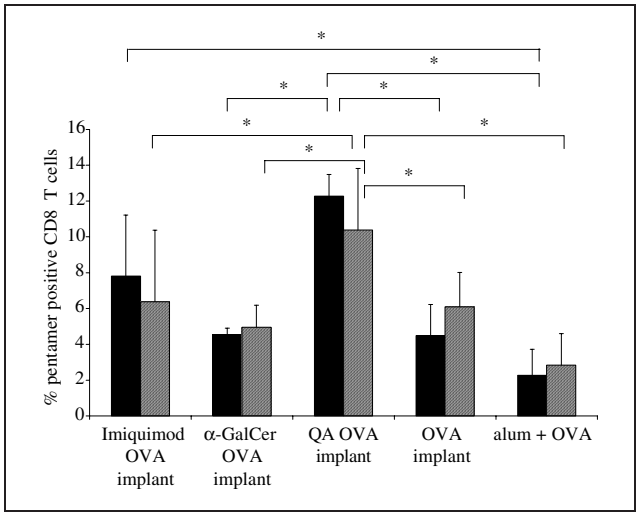

Fig. 4: Expansion of $CD8^+$ T cells recognising the CD8 epitope of OVA (SIINFEKL), as a percentage of total CD8 T cells. Black bars represent the results obtained from splenocytes; dashed bars represent the results obtained from lymphocytes. The graph shows data pooled from three independent experiments (n = 2–3 mice in each experiment) and is expressed as mean ± S.D. Statistical differences were determined using ANOVA followed by Tukey's pairwise comparison with $p \leq 0.05$ regarded as significantly different. Significant results are indicated by an*

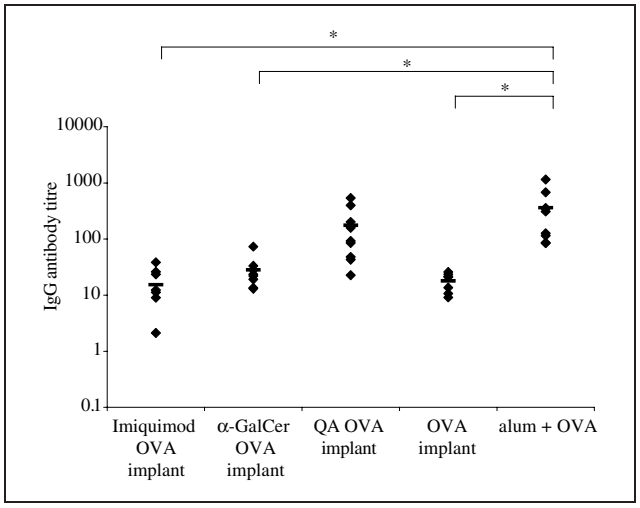

Fig. 5: OVA IgG antibodies determined using an OVA-specific ELISA. The diamonds represent the values obtained for each mouse; the lines represent the mean values for each group. The graph shows the results of three pooled experiments ($n = 2–3$ in each experiment). Statistical analysis was performed using ANOVA followed by Tukey's pairwises comparison. A p-value of $p \leq 0.05$ was regarded as significantly different, significant results are indicated by an*

cant when compared with all other vaccine groups (Fig. 4). In addition, the imiquimod OVA implant also promoted a strong expansion of $CD8^+$ T cells for both lymphocytes and splenocytes; however this difference was only significant for the splenocytes in comparison with the alum + OVA control group. Overall, the percentage of SIINFEKL positive $CD8^+$ T cells was slightly higher compared to the expansion of transgenic $CD8^+$ T cells, as all $CD8^+$ T cells were traced by the SIINFEKL positive cells whereas only the transgenic cell population was investigated in the first experiment. All implants resulted in the stimulation and expansion of SIINFEKL specific CD8 T cells which was at least as good as two immunisation of OVA in alum administered by subcutaneous injection.

2.4. Production of anti-OVA-IgG antibodies determined in serum samples

In addition to the investigation of the stimulation of cell-mediated immunity, the humoral immune response, represented by the production of specific anti-OVA-IgG antibodies was investigated (Fig. 5). As anticipated alum, which has been shown to induce good antibody responses (Gupta 1998), stimulated the highest antibody titres, at levels that were significantly higher than those stimulated by imiquimod OVA, α-GalCer OVA and OVA implant formulations. Even though the antibody response towards the QA OVA implant were found to be higher than those stimulated by the other adjuvant containing formulations and the OVA implant, these differences were not significant.

3. Discussion

Sustained release vaccines are an attractive alternative to traditional injectable vaccines, because they may be able to reduce the number of vaccinations needed to achieve protection. By reducing the number of vaccinations, patient compliance can be increased and a better acceptability of the vaccination concept can be achieved.

Indeed, this study demonstrates that lipid implant containing adjuvants can stimulate cellular and, to some extent, humoral immune responses. Nonetheless, differences in the ability to stimulate the immune system were observed between the formulations containing the different adjuvants. The strongest expansion of transgenic $CD8^+$ T cells and SIINFEKL-specific CD8 T cells was observed for implants containing the adjuvant QA, confirming that QA is a strong adjuvant capable of stimulating cell-mediated immunity. The results for the QA OVA implants in this study were in accordance with previous findings in our lab (Myschik et al. J. Drug Target., accepted for publication).

Surprisingly, neither the α-GalCer analogue nor imiquimod were able to stimulate immune responses comparable to those induced by the QA OVA implant. This finding was unexpected, as a strong stimulation of *i*NKT cells by α-GalCer was anticipated based on previous reports (Fujii et al. 2003), and some stimulation was observed by the α-GalCer analogue (Lee et al. 2006). Other studies have shown that both α-GalCer and imiquimod are potent immune response modifiers of peptide vaccines (Hermans et al. 2003; Johnston and Bystryn 2006; Johnston et al. 2007; Lang et al. 2006; Silk et al. 2004) or DNA vaccines (Thomsen et al. 2004).

As previously mentioned, imiquimod is commonly applied as a topical cream, and the subcutaneous administration in form of an injection of imiquimod is less common. To the best of our knowledge this is the first investigation of the delivery of either imiquimod or α-GalCer from a sustained release formulation. The doses of adjuvants in the implants were based on published studies using immediate release formulations given intravenously (i.v.) (Fujii et al. 2003; Hermans et al. 2003) or intraperitoneally (i.p.) (Chung et al. 2004). However, the rate of release of adjuvants from the implant formulation may be quite different compared to that of an immediate release vaccine. This may have resulted in the sustained release of sub-therapeutic levels of adjuvant from the implants. In addition the different chemical properties of the adjuvants, and the effect this would have on release, must be considered. Due to its branched sugar side chains, QA is water-soluble, and subsequently features greater hydrophilicity than the other adjuvants tested in this study. Therefore we assume that the release of QA could have taken place at a similar rate to the protein antigen whereas both imiquimod and α-GalCer possess higher hydrophobicity and may be released at a slower rate than OVA, thereby resulting in a reduced level of activation with these adjuvants.

As regards α-GalCer there is an added complication in that it has been reported that some cellular phospholipids also bind to the CD1d receptor (De Silva et al. 2002; Tsuji 2006), potentially affecting the affinity of other lipid molecules (Lawton and Kronenberg 2004). Phosphatidylcholine, which was used in the implants reported here, has been reported to bind to CD1d (Giabbai et al. 2005) but not to evoke an immune response. Therefore it is possible that in the system described here, competition for CD1d between phosphatidylcholine (4 mg per implant) and α-GalCer (1 μg per implant), may have taken place. Therefore, the choice of a different lipid, one with no affinity to CD1d, may lead to the development of a better formulation.

A study which focuses on the titration of the amount of imiquimod and α-GalCer in sustained release lipid implants may provide answers to these issues. The possible bell-shaped curve in cytokine-enhancing properties reported for imidazoquinolines (Thomsen et al. 2004), will have to be taken into consideration in future studies.

Furthermore, it may be necessary to determine the immune responses closer to the time of administration, because the peak immune response may have been missed as responses were determined 33 days after the initial administration in this study.

With regard to the stimulation of humoral immunity, OVA + alum was able to stimulate anti-OVA-IgG antibodies to a greater extent than any other formulation tested in this study. This finding was anticipated, given that alum has been reported to primarily induce the production of antibodies, but is unable to activate cell-mediated immunity (Gupta 1998). Cell-mediated immunity, however, is desirable for cancer vaccines or vaccines that target diseases caused by intracellular pathogens. Alum was inferior to the QA-containing formulation in relation to the expansion of $CD8^+$ T cells and SIINFEKL-specific $CD8^+$ T cells.

Considering the biocompatibility of the vaccines, all mice showed an increase in body weight during the course of the study. This indicates that the vaccines were well tolerated, and that no systemic toxicity effects, which would have caused weight loss, occurred. In addition, we were unable to detect adverse reactions to the anaesthetic or the implantation procedure, a finding supported by other studies performed earlier in our lab (Myschik et al., J. Drug Target., accepted for publication).

In conclusion, implants containing QA proved to be superior to those that contained either imiquimod or α-GalCer in stimulating the expansion of $CD8^+$ T cells and antibodies. These results indicate that further studies should be carried out to optimise imiquimod and α-GalCer lipid-based sustained release formulations.

4. Experimental

4.1. Materials

Cholesterol (purity approx. 95%) was obtained from Sigma-Aldrich, MO, USA. Phosphatidylcholine from egg yolk (PC) was purchased from Northern Lipids INC., Vancouver, Canada. Purified saponin Quil-A™ (lyophilised powder) was obtained from Brenntag Biosector, Frederikssund, Denmark. The α-GalCer analogue was kindly provided by IRL, Wellington, New Zealand. Imiquimod was obtained from Topharman (Shanghai, China). Albumin from chicken egg (OVA), phosphate buffered saline pH 7.4 (0.01 M) were obtained from Sigma-Aldrich, MO, USA. Chloroform (purity 99–99.4%) was supplied by Merck, Darmstadt, Germany. Distilled deionised water having a conductivity of less than 0.1 μS (Milli-Q Water systems, Millipore, MA, USA) was used in this study. Alu-Gel-S (SERVA Electrophoresis GmbH, Heidelberg, Germany) was used for the preparation of ovalbumin in alum.

The following agents were used for analysis of cells by flow cytometry: anti-CD16/CD32 antibody (2.4G2 Fc block), CD4-FITC, Vα2-PE, Vβ5.1-biotin, CD8-APC, CD8-PE, CD4-biotin, anti-CD3 (all purchased from BD Pharmingen, San Diego, CA, USA). Cells stained with biotinylated anti-mouse antibodies were also stained with streptavidin conjugated to PerCP to visualise the biotinylated antibodies. SIINFEKL specific pentamer labelled with phycoerythrin (PE) (PROIMMUNE, Oxford, United Kingdom) was used for SIINFEKL-specific staining in accordance with the manufacturer's instructions. IL-2 was kindly provided by the Malaghan Institute of Medical Research, Wellington, New Zealand.

Single-cell suspensions were prepared in sterile complete Iscove's Modified Dulbecco's Medium (cIMDM; IMDM supplemented with 5% foetal bovine serum, 1% penicillin/streptomycin, 1% glutamax and 0.01% 2-mercaptoethanol (all from Invitrogen, Carlsbad, CA, USA)).

Di-sodium hydrogen orthophosphate (anhydrous), potassium chloride, sodium hydrogen carbonate and potassium dihydrogen orthophosphate were used in the OVA ELISA and purchased from BDH Laboratory Supplies (Poole, UK). Sodium carbonate was obtained from Merck (Darmstadt, Germany). Sodium chloride, sodium hydroxide and hydrogen peroxide 30% were purchased from Univar, Ajax Finechem (Auckland, New Zealand). Polyoxyethylene sorbitan monolaurate (Tween 20) and stop reagent for TMB substrate were supplied by Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Bovine serum albumin (BSA), Fraction V, low endotoxicity, was obtained from GIBCO, Invitrogen Corporation, New Zealand.

4.2. Methods

4.2.1. Preparation of implants

The preparation of all implants was performed as described earlier as a freeze-dried lipid mixture of cholesterol and phospholipid (Myschik, et al. 2008a) at a ratio of cholesterol to phospholipid of 2 : 8 for implants containing imiquimod or α-GalCer, and a ratio of 0.2 : 2 : 7.8 (QA : CHOL : PC) for implants containing QA. A punch and die system of 2 mm in diameter was used for the compression of the implants and a mass of 0.2 tons was applied (Carver Laboratory Press, Model 3392, Fred S. Carver Inc., Wisconsin, USA). Prior to compression, the lipid powder mixture was frozen at −80 °C to allow better compressibility. All implants contained approximately 20 μg OVA. The release of the antigen was slowed down by the addition of 40% excess crystalline cholesterol (by weight) admixed by light trituration in a mortar to the lipid powder mixture prior to compression as reported elsewhere (Myschik et al., J. Drug Target., accepted for publication). Implants had a cylindrical shape and the final weight of each implant was approximately 8.9–9.1 mg. All formulations were prepared and analysed in triplicate.

4.2.2. Transmission electron microscopy

In order to investigate the formation of colloidal particles from the implants, these were placed into vials containing 400 μl PBS buffer and were kept at 37 °C in a water bath. Samples (50 μl) were taken at various time points (after 1 h, 4 h, 8 h, 24 h, 48 h, 5 days and 9 days) and the volume withdrawn was replaced with the same volume of fresh PBS buffer. Carbon-coated copper grids were glow-discharged (Edwards E306A Vacuum Coater, England). Ten μl of each sample were adsorbed onto a grid and excess sample was removed using filter paper (Whatman International Ltd. Maidstone, England). Grids were negatively stained with 10 μl of 2% phosphotungstic acid (Agar Scientific Limited, Essex, England), pH 5.2.

4.3. Animals

C57Bl/6 mice from 6–8 weeks of age were bred at the HTRU and kept under specific pathogen-free (SPF) conditions during the course of this study. Transgenic mice expressing either H-$2K^b$ restricted $CD8^+$ T cells specific for the OVA peptide SIINFEKL ($OVA_{257-264}$), termed OT-I transgenic mice, and mice expressing a high proportion of T cell receptors (TCRs) specific for the CD4 epitope of $OVA_{323-339}$, termed OT-II transgenic mice, were held under the same conditions. Mice had access to water and food available *ad libitum*. Approval for the experiments was obtained from the University of Otago Animal Ethics Committee.

4.3.1. Adoptive transfer of OVA-specific TCR transgenic T cells

OT-I and OT-II transgenic mice were sacrificed by cervical dislocation and spleens, axial, brachial and mesenteric lymph nodes were harvested. To obtain lymphocyte and splenocyte cell suspension, lymph nodes and spleens were passed through sterile gauze. Red blood cells were deleted using lysis buffer (0.17 M Tris-HCl and 0.16 M ammonium chloride at a ratio of 1 : 9, pH 7.65). 8×10^6 cells/ml were resuspended as single cell-suspensions in cIMDM and were counted after centrifugation (approx. $260 \times g$ for 8 min). Phase-contrast microscopy was applied to exclude dead cells after colouring cells with trypan blue stain 0.4% (Invitrogen, USA). In the final step cells were resuspended at a concentration of 8×10^6 cells/ml in sterile PBS. 500 μl of transgenic cell suspension were adoptively transferred into C57Bl/6 mice via tail vein injection one day prior to the immunisation.

4.3.2. Immunisation protocol

One day after the adoptive transfer of transgenic T cells, mice were anaesthetised using halothane and a small cut was made on the shaved back of the mice. A trochar was carefully inserted into the cut through which the implants were administered under aseptic conditions. The wound was subsequently closed using a Michel clip. Mice were immunised with an implant containing either 10 μg imiquimod/20 μg OVA, 1 μg α-galactosylceramide/20 μg OVA, 100 μg QA/20 μg OVA, or an implant containing no adjuvant/20 μg (OVA implant). One group of mice was inoculated with an injectable vaccine of 10 μg OVA in alum. A booster immunisation was given to mice which received OVA in alum on day 14. All mice were challenged on day 29 with 10 μg OVA in PBS and sacrificed 4 days later. Spleens, lymph nodes and blood samples were harvested for analysis.

4.4. Flow cytometry

Cells were incubated with anti-CD16/CD32 antibody (2.4G2 Fc Block) and washed in fluorescence-activated cell sorting (FACS) buffer (PBS containing 1% bovine serum albumin and 0.01% sodium azide) to block unspecific binding. A combination of CD4-FITC, Vα2-PE, Vβ5.1-biotin, CD8-APC or CD8-FITC, SIINFEKL-pentamer, CD-25 mAb were added to the FACS buffer according to the manufacturer's instructions and incubated in the dark. To visualise biotinylated antibodies samples were subsequently stained with streptavidin conjugated to PerCP. The PE-labelled SIINFEKL-

pentamer allows for the detection of all specific $CD8^+$ T cells responding to the SIINFEKL peptide. All cell suspensions were analysed using flow cytometry (FACScalibur, Becton Dickinson, Franklin Lakes, NJ, USA) and data was processed using CellQuest Pro (Becton Dickinson).

4.5. OVA-specific antibody production in vitro (anti-OVA IgG ELISA)

Blood samples were collected from all animals through the inferior vena cava and spun for 10 minutes at $2570 \times g$ (Centrifuge 5417 C, Eppendorf, Hamburg, Germany) to separate the serum from the red blood cells. The fraction containing the serum was collected and stored in a freezer at −20 °C until required. 96-well ELISA plates (BD Biosciences, Bedford, MA, USA) were coated with 100 µl of a 10 µg/ml solution of OVA in coating buffer and incubated over night. After washing, wells were blocked with 150 µl blocking buffer. Blocking buffer was discarded and serum samples were added to the wells in a 1 in 10 dilution and a 1 in 2 dilution to each successive well. After incubation for 2 h at room temperature, wells were washed and goat-anti-mouse IgG (H + L) HRP conjugate (Zymed, San Francisco, CA, USA) was added to each well for 1 h. Thereafter, plates were washed and HRP substrate containing 1 µl/ml hydrogen peroxide 30% was added. After incubation of 10 min in the dark the reaction was stopped using 2 mM sodium azide solution. Plates were analysed using a Spectra Max 340 Plate Reader (Molecular Devices) at a wavelength of 414 nm.

4.6. Monitoring of mice and tolerance of vaccine formulations

Mice were monitored closely after adoptive transfer and the injection/implantation procedure. Regular weight checks and verification of water and food intake were carried out to ensure the well-being of the animals and the tolerance of the different vaccine formulations.

4.7. Statistical analysis

Where applicable, results are expressed as mean +/− S.D. Statistical analysis was carried out by one-way analysis of variance (ANOVA) with a confidence interval of 95% using SPSS® 14.0, Release 14.0.0 (SPSS Inc., Chicago, IL, USA) followed by post-hoc analysis using Tukey's pairwise comparison.

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References

Akira S (2003) Mammalian Toll-like receptors. Curr Opin Immunol 15: 5–11.

Barr IG, Sjolander A, Cox JC (1998) ISCOMs and other saponin based adjuvants. Adv Drug Deliv Rev 32: 247–271.

Behboudi S, Morein B, Villacres-Eriksson MC (1999) Quillaja Saponin Formulations that Stimulate Proinflammatory Cytokines Elicit a Potent Acquired Cell-Mediated Immunity. Scand J Immunol 50: 371–377.

Borg NA, Wun KS, Kjer-Nielsen L, Wilce MCJ, Pellicci DG, Koh R, Besra GS, Bharadwaj M, Godfrey DI, McCluskey J, Rossjohn J (2007) CD1d-lipid-antigen recognition by the semi-invariant NKT T-cell receptor. Nature 448 (7149): 44–49.

Bramwell VW, Perrie Y (2005) Particulate delivery systems for vaccines. Crit Rev Ther Drug Carrier Syst 22: 151–214.

Brutkiewicz RR (2006) CD1d Ligands: The Good, the Bad, and the Ugly. J Immunol 177: 769–775.

Burstein HJ, Shea CM, Abbas AK (1992) Aqueous antigens induce in vivo tolerance selectively in IL-2 and IFN-y producing (Th1) cells. J Immunol 148: 3687–3691.

Chung Y, Chang W-S, Kim S, Kang C-Y (2004) NKT cell ligand α-galactosylceramide blocks the induction of oral tolerance by triggering dendritic cell maturation. Eur J Immunol 34: 2471–2479.

De Silva AD, Park JJ, Matsuki N, Stanic AK, Brutkiewicz RR, Medof ME, Joyce S (2002) Lipid protein interactions: the assembly of CD1d1 with cellular phospholipids occurs in the endoplasmic reticulum. J Immunol 168: 723–733.

Demana PH, Davies NM, Hook S, Rades T (2005) Quil A-lipid powder formulations releasing ISCOMs and related colloidal structures upon hydration. J Control Release 103: 45–59.

Fujii S-i, Shimizu K, Smith C, Bonifaz L, Steinman RM (2003) Activation of Natural Killer T Cells by α-Galactosylceramide Rapidly Induces the Full Maturation of Dendritic Cells In Vivo and Thereby Acts as an Adjuvant for Combined CD4 and CD8 T Cell Immunity to a Coadministered Protein. J Exp Med 198: 267–279.

Giabbai B, Sidobre S, Crispin MDM, Sanchez-Ruìz Y, Bachi A, Kronenberg M, Wilson IA, Degano M (2005) Crystal Structure of Mouse CD1d Bound to the Self Ligand Phosphatidylcholine: A Molecular Basis for NKT Cell Activation. J Immunol 175: 977–984.

Giaccone G, Punt CJA, Ando Y, Ruijter R, Nishi N, Peters M, von Blomberg BME, Scheper RJ, van der Vliet HJJ, van den Eertwegh AJM, Roelvink M, Beijnen J, Zwierzina H, Pinedo HM (2002) A Phase I Study of the Natural Killer T-Cell Ligand {alpha}-Galactosylceramide (KRN7000) in Patients with Solid Tumors. Clin Cancer Res 8: 3702–3709.

Gupta AK, Cherman AM, Tyring SK (2004) Viral and Nonviral Uses of Imiquimod: A Review. J Cutan Med Surg 8: 338–352.

Gupta RK (1998) Aluminium compounds as vaccine adjuvants. Adv Drug Deliv Rev 32: 155–172.

Guse C, Koennings S, Kreye F, Siepmann F, Goepferich A, Siepmann J (2006a) Drug release from lipid-based implants: Elucidation of the underlying mass transport mechanisms. Int J Pharm 314: 137–144.

Guse C, Koennings S, Maschke A, Hacker M, Becker C, Schreiner S, Blunk T, Spruss T, Goepferich A (2006b) Biocompatibility and erosion behaviour of implants made of triglycerides and blends with cholesterol and phospholipids. Int J Pharm 314: 153–160.

Hayakawa Y, Godfrey DI, Smyth MJ (2004) α-Galactosylceramide: Potential Immunomodulatory Activity and Future Application. Curr Med Chem 11: 241–252.

Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, Hoshino K, Horiuchi T, Tomizawa H, Takeda K, Akira S (2002) Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat Immunol 3: 196–200.

Hermans IF, Silk JD, Gileadi U, Salio M, Mathew B, Ritter G, Schmidt R, Harris AL, Old L, Cerundolo V (2003) NKT Cells Enhance $CD4^+$ and $CD8^+$ T Cell Responses to Soluble Antigen In Vivo through Direct Interaction with Dendritic Cells. J Immunol 171: 5140–5147.

Herrmann S, Winter G, Mohl S, Siepmann F, Siepmann J (2007) Mechanisms controlling protein release from lipidic implants: Effects of PEG addition. J Control Release 118: 161–168.

Ishikawa A, Motohashi S, Ishikawa E, Fuchida H, Higashino K, Otsuji M, Iizasa T, Nakayama T, Taniguchi M, Fujisawa T (2005) A Phase I Study of {alpha}-Galactosylceramide (KRN7000)-Pulsed Dendritic Cells in Patients with Advanced and Recurrent Non-Small Cell Lung Cancer. Clin Cancer Res 11: 1910–1917.

Johansson M, Lövgren-Bengtsson K (1999) Iscoms with different quillaja saponin components differ in their immunomodulating activities. Vaccine 17: 2894–2900.

Johnston D, Bystryn J-C (2006) Topical imiquimod is a potent adjuvant to a weakly-immunogenic protein prototype vaccine. Vaccine 24: 1958–1965.

Johnston D, Zaidi B, Bystryn J-C (2007) TLR7 imidazoquinoline ligand 3M-019 is a potent adjuvant for pure protein prototype vaccines. Cancer Immunol Immunother 56: 1133–1141.

Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo E, Koseki H, Taniguchi M (1997) CD1d-Restricted and TCR-Mediated Activation of V{alpha}14 NKT Cells by Glycosylceramides. Science 278: 1626–1629.

Kensil CR (1996) Saponins as vaccine adjuvants. Crit Rev Ther Drug Carrier Syst 13: 1–55.

Kensil CR, Patel U, Lennick M, Marciani D (1991) Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria* Molina Cortex. J Immunol 146: 431–437.

Kersten GF, Spiekstra A, Beuvery EC, Crommelin DJA (1991) On the structure of immune-stimulating saponin-lipid complexes (iscoms). Biochim Biophys Acta 1062: 165–171.

Khan MZI, Tucker IG, Opdebeeck JP (1991) Cholesterol and lecithin implants for sustained release of antigen: release and erosion in vitro, and antibody response in mice. Int J Pharm 76: 161–170.

Khan MZI, Tucker IG, Opdebeeck JP (1993) Evaluation of cholesterol-lecithin implants for sustained delivery of antigen: release in vivo and single-step immunisation of mice. Int J Pharm 90: 255–262.

Koennings S, Tessmar J, Blunk T, Goepferich A (2007) Confocal Microscopy for the Elucidation of Mass Transport Mechanisms Involved in Protein Release from Lipid-based Matrices. Pharm Res 24: 1325–1335.

Lang GA, Exley MA, Lang ML (2006) The CD1d-binding glycolipid α-galactosylceramide enhances humoral immunity to T-dependent and T-independent antigen in a CD1d-dependent manner. Immunology 119: 116–125.

Lawton AP, Kronenberg M (2004) The Third Way: Progress on pathways of antigen processing and presentation by CD1. Immunol Cell Biol 82: 295–306.

Lee A, Farrand KJ, Dickgreber N, Hayman CM, Jurs S, Hermans IF, Painter GF (2006) Novel synthesis of [alpha]-galactosyl-ceramides and confirmation of their powerful NKT cell agonist activity. Carbohydr Res 341: 2785–2798.

Lendemans DG (2006) Novel cationic preparations of ISCOMs as vaccine carriers. PhD thesis, University of Otago, Dunedin, New Zealand.

Marciani DJ (2003) Vaccine adjuvants: role and mechanisms of action in vaccine immunogenicity. Drug Discov Today 8: 934–943.

Mohl S, Winter G (2004) Continuous release of rh-interferon [alpha]-2a from triglyceride matrices. J Control Release 97: 67–78.
Naidenko OV, Koezuka Y, Kronenberg M (2000) CD1-mediated antigen presentation of glycosphingolipids. Microbes Infect 2: 621–631.
Natori T, Morita M, Akimoto K, Koezuka Y (1994) Agelasphins, novel antitumor and immunostimulatory cerebrosides from the marine sponge *Agelas mauritianus*. Tetrahedron 50: 2771–2784.
Parekh VV, Singh AK, Wilson MT, Olivares-Villagomez D, Bezbradica JS, Inazawa H, Ehara H, Sakai T, Serizawa I, Wu L, Wang C-R, Joyce S, Van Kaer L (2004) Quantitative and Qualitative Differences in the In Vivo Response of NKT Cells to Distinct {alpha}- and {beta}-Anomeric Glycolipids. J Immunol 173: 3693–3706.
Rönnberg B, Fekadu M, Morein B (1995) Adjuvant activity of non-toxic *Quillaja saponaria* Molina components for use in ISCOM matrix. Vaccine 13: 1375–1382.
Schiller M, Metze D, Luger TA, Grabbe S, Gunzer M (2006) Immune response modifiers – mode of action. Exp Dermatol 15: 331–341.
Silk JD, Hermans IF, Gileadi U, Chong TW, Shepherd D, Salio M, Mathew B, Schmidt RR, Lunt SJ, Williams KJ, Stratford IJ, Harris AL, Cerundolo V (2004) Utilizing the adjuvant properties of CD1d-dependent NK T cells in T cell-mediated immunotherapy. J Clin Invest 114: 1800–1811.
Soltysik S, Wu J-Y, Recchia J, Wheeler DA, Newman MJ, Coughlin RT, Kensil CR (1995) Structure/function studies of QS-21 adjuvant: assessment of triterpene aldehyde and glucuronic acid roles in adjuvant function. Vaccine 13: 1403–1410.
Stanley MA (2002) Imiquimod and the imidazoquinolones: mechanism of action and therapeutic potential. Clin Exp Dermatol 27: 571–577.
Thomsen LL, Topley P, Daly MG, Brett SJ, Tite JP (2004) Imiquimod and resiquimod in a mouse model: adjuvants for DNA vaccination by particle-mediated immunotherapeutic delivery. Vaccine 22: 1799–1809.
Tomai MA, Imbertson LM, Stanczak TL, Tygrett LT, Waldschmidt TJ (2000) The Immune Response Modifiers Imiquimod and R-848 Are Potent Activators of B Lymphocytes. Cell Immunol 203: 55–65.
Tsuji M (2006) Glycolipids and phospholipids as natural CD1d-binding NKT cell ligands. Cell Mol Life Sci 63: 1889–1898.
Vogelhuber W, Magni E, Mouro M, Spruß T, Guse C, Gazzaniga A, Goepferich A (2003) Monolithic triglyceride matrices: a controlled-release system for proteins. Pharm Dev Technol 8: 71–79.
Wagner TL, Ahonen CL, Couture AM, Gibson SJ, Miller RL, Smith RM, Reiter MJ, Vasilakos JP, Tomai MA (1999) Modulation of TH1 and TH2 Cytokine Production with the Immune Response Modifiers, R-848 and Imiquimod. Cell Immunol 191: 10–19.
Walduck AK, Opdebeeck JP, Benson HE, Prankerd R (1998) Biodegradable implants for the delivery of veterinary vaccines: design, manufacture and antibody response in sheep. J Control Release 51: 269–280.
Weeratna RD, Makinen SR, McCluskie MJ, Davis HL (2005) TLR agonists as vaccine adjuvants: comparison of CpG ODN and Resiquimod (R-848). Vaccine 23: 5263–5270.
Zinkernagel RM, Ehl S, Aichele P, Oehen S, Kuendig T, Hengartner H (1997) Antigen localisation regulates immune responses in a dose- and time-dependent fashion: a geographical view of immune reactivity. Immunol Rev 156: 199–209.