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Mannosylated liposomes as antigen delivery vehicles for targeting to dendritic cells

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Abstract

The immune stimulating ability of mannosylated liposomes containing FITC-ovalbumin as a model antigen and displaying either a branched tri-mannose or a mono-mannose ligand on the liposome surface was investigated in human monocyte-derived dendritic cells (MoDCs) and murine bone-marrow-derived dendritic cells (BMDCs). Uptake of liposomes, dendritic cell activation and proliferation of $CD8^+$ T cells from OT-I transgenic mice were determined by flow cytometry. Uptake of liposomes displaying the tri-mannose ligand was enhanced in human MoDCs compared with both non-mannosylated liposomes and liposomes displaying mono-mannose ligands. However, this increased uptake did not result in an increase in expression of CD80 or CD86 on the surface of the MoDCs. In contrast, neither tri-mannose- nor mono-mannose-containing liposomes were taken up by murine BMDCs to a greater extent than non-mannose-containing liposomes. The expression of CD86 and CD40 on the surface of BMDCs was not increased after exposure to mannosylated liposomes and BMDCs incubated with mannosylated liposomes were not able to stimulate proliferation of $CD8^+$ T cells to any greater extent than BMDCs incubated with non-mannosylated liposomes. These findings suggest that while mannose-containing ligands can enhance the uptake of antigen-containing liposomes by some dendritic cells, important differences in the affinity of carbohydrate-binding receptors for mannose-containing ligands do exist between species. In addition, the increase in uptake of antigen by dendritic cells using mannosylated liposomes does not necessarily result in enhanced dendritic cell activation.

Introduction

Dendritic cells are professional antigen-presenting cells central to the initiation of immune responses against a wide variety of pathogens. They also have a role in tumour immunotherapy and in the development of tolerance to self-antigens (Banchereau & Steinman 1998). Immature dendritic cells found in non-lymphoid tissue are highly phagocytic and well adapted to monitoring their environment for potential pathogens (Guermonprez et al 2002). Upon recognition of a potential threat or danger signal, dendritic cells begin a maturation process that involves the down-regulation of phagocytic capabilities and the up-regulation of antigen processing and presentation capabilities (Banchereau et al 2000, Bhardwaj 2001). Maturing dendritic cells migrate to the lymphoid tissue where they are able to interact with large numbers of immature B and T lymphocytes (Miller et al 2004). Dendritic cells present processed antigens on their surface to T cells in conjunction with MHC molecules for recognition by the T cell receptor along with co-stimulatory molecules, such as CD80 and CD86, required for appropriate activation of naïve T cells (Banchereau et al 2000). While naïve B lymphocytes are able to recognise and respond to antigen without dendritic cell presentation, naïve T cells cannot. In fact, without the appropriate dendritic cell presentation and co-stimulation, tolerance may result (Mahnke et al 2003; Steinman et al 2003).

Dendritic cells have evolved numerous mechanisms for sampling their environment, including macropinocytosis, phagocytosis and receptor-mediated phagocytosis via non-specific pattern recognition receptors including Toll-like receptors (TLR) and

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the C-type lectins (Banchereau et al 2000, Guermonprez et al 2002). The C-type lectin family of receptors are a group of structurally related surface-bound receptors and soluble proteins. The main feature linking this diverse family of receptors is the presence of one or more common folds in the protein tertiary structure called the carbohydrate recognition domain (CRD) (Weis et al 1991). To bind to the CRD, a carbohydrate must first interact with the primary binding site of the receptor. However, ligand specificity is determined by interactions between hydroxyl groups on the carbohydrate and amide side groups present within the fold. Additional specificity is conferred by electrostatic interactions and hydrogen bonding on the surface of the protein (Kolatkar & Weis 1996; Ng & Weis 1997). The entire carbohydrate structure therefore plays a role in determining the specificity and affinity of the interaction with the CRD and factors such as degree of carbohydrate branching, spacing, and multivalency are important (Taylor & Drickamer 1993; Geijtenbeek et al 2004; McGreal et al 2004).

Of the C-type lectins known to date, several, including the mannose receptor (MR), dendritic, cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) and Langerin, display affinity for mannose-containing ligands, although the specificity of ligand binding to these receptors differ markedly. The MR, for instance, binds to end-standing mono- and di-mannose residues, whereas DC-SIGN has a higher affinity for internal mannose structures containing a minimum of three branched mannose residues and end-standing di-mannose-containing structures (Feinberg et al 2001; Frison et al 2003). DC-SIGN also has a higher affinity for fucose-containing structures compared with the MR (Appelmelk et al 2003).

The downstream effects from binding to C-type lectins are not well characterised. Some studies report that C-type lectins primarily function as receptors for the recognition of pathogens and for antigen internalisation (Hiltbold et al 2000; Heystek et al 2002; Napper & Taylor 2004). Other reports suggest that C-type lectins are capable of intracellular signalling, which may interfere with dendritic cell activation arising through TLR ligation (Nigou et al 2001; Geijtenbeek et al 2003). It has been suggested that the affinity of ligand binding to the C-type lectins may influence the outcome of this interaction. More research is still needed, therefore, to investigate the effect the nature of the mannose-containing ligand has on immune system activation.

Previous work in our laboratory has shown that liposomes containing a tri-mannose phospholipid ligand are a useful delivery system for targeting antigens to human monocyte-derived dendritic cells (MoDCs) (Copland et al 2003). Mannosylated liposomes containing fluorescently labelled ovalbumin protein as a model antigen were taken up by MoDCs to a greater extent than non-targeted liposomes. This is in agreement with other work in the literature that found targeted delivery of antigen to dendritic cells leads to enhanced antigen uptake (Stahl et al 1980; Garcon et al 1988; Engering et al 1997; Davis et al 2002). In our previous study, mannosylated liposomes containing tetanus toxoid were also able to stimulate greater proliferation of primed T cells than if the antigen was delivered in non-targeted liposomes or in solution.

The work presented here expands on our previous study using mannosylated liposomes as an antigen delivery system by investigating the influence of the degree of mannosylation on the uptake of liposomes by dendritic cells and subsequent dendritic cell activation. As multivalent mannosylated ligands may influence if dendritic cells become activated or if immune function is suppressed, the nature of the mannose ligand and its influence on uptake and dendritic cell activation was also studied using a ligand with a single mannose head group compared with a branched tri-mannose head group. In addition, the uptake of mannosylated liposomes and the resulting activation of dendritic cells in both MoDCs and murine bone-marrow-derived dendritic cells (BMDCs) were compared to investigate the similarities or differences between these two important dendritic cell models. Finally, whether dendritic cells activated with antigen-loaded, mannosylated liposomes were able to stimulate activation of $CD8^+$ T cells in-vitro was investigated.

Materials and Methods

Antibodies

Anti-mouse 2.4G2, CD11c, CD86, CD40 and Vα2, and anti-human CD1a and CD80 were purchased from BD Pharmingen (USA) and were used according to the manufacturer's instructions. Anti-human CD86 was purchased from Serotec (UK) and also used as directed by the manufacturer.

Liposome preparation

Liposome formulations were prepared by hydration of thin lipid films following methods previously described (Copland et al 2003). Briefly, 50 mg of total lipid (L-α-phosphatidylcholine (from egg yolk; Sigma), plus either the trimannose-dipalmitoylphosphatidylethanolamine (8-[carboxy-2-(1,2-dihexadecanoyl-sn-glycero-3-sodio-phospho)ethanolamido]octyl (α-D-mannopyranosyl)-(1$\rightarrow$3)-[(α-D-mannopyranosyl)-(1$\rightarrow$6)]-α-D-mannopyranoside, M_3-DPPE) or the monomannose-dipalmitoylphosphatidylethanolamine(8-[carboxy-2-(1,2-dihexadecanoyl-sn-glycero-3-sodio-phospho)ethanolamido]octyl α-D-mannopyranoside, M_1-DPPE) (Becker et al 1999) were solubilised in a mixture of methanol and chloroform (1:5 ratio) in a round-bottomed flask. The amount of M_3-DPPE and M_1-DPPE was varied between 0% and 20% of the total lipid weight. The solvent was removed by evaporation under vacuum at 45°C leaving a thin lipid film on the wall of the flask. The lipid film was then flushed with nitrogen gas to ensure removal of all residual solvent. A 1-mL volume of 10 mM HEPES buffer, pH 7.4, containing 10 mg mL^{-1} ovalbumin (chicken egg ovalbumin, grade VI; Sigma) conjugated to fluorescein 5-isothyocyanate (FITC, isomer 1; Sigma), using a method previously described (Copland et al 2003), was added to the lipid

film. Hydration was aided by handshaking of the flask, resulting in the formation of large multilamellar vesicles. Vesicle size and lamellarity was reduced by repeated extrusion through 800-nm polycarbonate membranes (Nucleopore Corp, Pleasanton, CA, USA) using a 10-mL extruder (Lipex Biomembranes Inc., Vancouver, Canada) pressurised with nitrogen gas. Unentrapped protein was removed by multiple centrifugation of the liposome dispersion (14 000 *g* for 30 min) and washing with HEPES buffer.

Liposome characterisation

The size distribution of the liposomes when dispersed in 10 mM Hepes buffer, pH 7.4, was measured by photon correlation spectroscopy (Zetasizer 3000; Malvern). Protein incorporation within liposomes was determined by lysing the liposomes with phosphate buffer, pH 6.5, containing 5% Triton X-100 and measuring the fluorescence of the resulting solution (Shimadzu RF-540, $\lambda_{excitation}$ 490 nm, $\lambda_{emission}$ 520 nm). Fluorescence measurements were repeated periodically over 14 days to evaluate the stability of the liposome membrane with regards to retention of entrapped protein over time.

The presence of accessible mannose residues on the surface of the mannosylated liposomes was determined by measuring an increase in the mean particle size of liposome dispersions after the addition of concanavalin A (Con A). Ten microlitres of liposome dispersion was added to 3 mL of borate buffer, pH 8, containing 0.001 M $CaCl_2$ and the particle size distribution measured every 5 min (Zetasizer 3000; Malvern). Con A was added to a final concentration of 8 μg mL^{-1} after 20 min and particle size measurements continued for a further 70 min.

Generation of human monocyte derived dendritic cells

MoDCs were prepared from the blood of healthy human subjects obtained by venepuncture and prepared following the method previously described (Copland et al 2003). Briefly, peripheral blood lymphocytes were isolated from whole blood over a density gradient (Ficoll-Paque PLUS; Amersham Biosciences) and the resulting cells suspended at a concentration of 4×10^6 cells/mL in RPMI 1640 supplemented with 2% fetal bovine serum, 100 U mL^{-1} penicillin–100 μg mL^{-1} streptomycin and 2 mM GlutaMax–1, all from Invitrogen Corporation (complete RPMI, cRPMI). Cells were incubated at 37°C, 5% CO_2 for 90 min then non-adherent cells removed by gently pipetting and removing the cell supernatant. cRPMI supplemented with hGM-CSF (Leucomax; Sandoz) and hIL-4 (R & D Systems, MN) both at a final concentration of 50 ng mL^{-1}, was added to the remaining adherent cells and these were cultured at 37°C, 5% CO_2 to allow differentiation into MoDC. On the third day, 1 mL of supernatant was removed from each well and replaced with 1 mL of fresh cRPMI containing hGM-CSF and hIL-4. Cells were harvested on day 6 and were washed twice in fresh cRPMI before use. Experiments using cells obtained from human blood were carried out under the approval of the University of Otago Ethics Committee.

Generation of murine bone-marrow-derived dendritic cells

C57Bl/6J mice were bred and maintained in micro-isolators under specific-pathogen-free (SPF) conditions at the Hercus Tairei Resource Unit, Dunedin, New Zealand. All animal experiments were approved by the University of Otago Animal Ethics Committee.

Bone marrow was removed from the femurs of adult female C57Bl/6J mice by flushing with Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 5% fetal bovine serum, 100 U mL^{-1} penicillin–100 μg mL^{-1} streptomycin, 2 mM GlutaMax-1, and 55 μM 2-mercaptoethanol (Invitrogen Corporation) (complete IMDM, cIMDM). The red blood cells were lysed and the remaining cells re-suspended at 1×10^6 cells/mL in cIMDM supplemented with 20 ng mL^{-1} granulocyte macrophage colony stimulating factor (GM-CSF) (kindly gifted by Dr G Buchan, Department of Microbiology and Immunology, University of Otago, New Zealand). Cells were incubated at 37°C and 5% CO_2 to allow differentiation into BMDCs. On day 4, the entire supernatant was removed gently from each well and replaced with 5 mL of fresh cIMDM containing 10 ng mL^{-1} GM-CSF. Cells were harvested on day 6 and were washed twice in fresh cIMDM before use.

Uptake of liposomes by dendritic cells and dendritic cell activation

To investigate the ability of dendritic cells to take up and be activated by the different liposome formulations, 5 μL (250 μg total lipid) of the washed and re-suspended FITC-ovalbumin-containing liposome dispersions were added to 5×10^5 MoDCs or BMDCs suspended in 1 mL of complete media (cRPMI or cIMDM, respectively) and incubated for 2 days at 37°C and 5% CO_2. Previous experiments investigating the kinetics of liposome uptake and dendritic cell activation have shown that this time point is optimal for simultaneous determination of both parameters under the conditions used here (data not shown). Uptake of liposomes was determined by measuring the cell-associated FITC fluorescence of dendritic cells ($CD1a^+$ MoDCs and $CD11c^+$ BMDCs) by flow cytometry (FACScalibur; Becton-Dickinson). The fluorescence intensity of dendritic cells not pulsed with liposome formulations was also determined by flow cytometry and used as a control. We have previously used confocal microscopy and comparisons with levels of FITC fluorescence in cells incubated at 4°C to confirm that liposomes are actually taken up into cells and are not merely associated with the cell surface (Copland et al (2003) and data not shown).

Activation of dendritic cells was determined by staining the cells with antibodies against appropriate surface-activation markers (i.e. CD80 and CD86 for MoDCs and CD86 and CD40 for BMDCs) and analysing the dendritic cell marker-positive cells by flow cytometry. In all dendritic cell activation experiments, lipopolysaccharide (LPS, $10\,\mu g\,mL^{-1}$) was included as a positive control. All data were analysed using CellQuest Pro software (Becton-Dickinson).

T cell proliferation

The ability of liposome-pulsed dendritic cells to stimulate $CD8^+$ T cell proliferation in-vitro was investigated using splenocytes isolated from OT-I transgenic mice using a method previously described (Demana et al 2004). OT-I transgenic mice have been genetically engineered to express receptors for minimal CD8 epitopes from chicken egg ovalbumin on their T cells and as such can respond to ovalbumin antigens both in-vitro and in-vivo without the need for prior priming (Carbone & Bevan 1989). OT-I transgenic mice were bred and maintained in micro-isolators under specific-pathogen-free (SPF) conditions at the Hercus Tairei Resource Unit, Dunedin, New Zealand. Briefly, splenocytes from an OT-I transgenic mouse were isolated and stained with 5- (and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) and then incubated together with pulsed and washed BMDCs at a dendritic cell:T cell ratio of 1:25. Splenocytes were also incubated together with BMDCs not pulsed with any formulation and with BMDCs pulsed with LPS ($10\,\mu g\,mL^{-1}$) as negative and positive controls, respectively. CFSE staining in $V\alpha2^+$ $CD8^+$ OT-I transgenic splenocytes was determined after 72 h by flow cytometry.

Statistical analysis

Data is represented as the mean ± s.d. of the value of the mean. Student's *t*-test was used to test the significance of the difference between two means. Analysis of variance was used when more than two means were compared. $P < 0.05$ was considered to be statistically significant.

Results and Discussion

Liposome characterisation

Physical characterisation of the liposome dispersions containing mannosylated phospholipids showed that as the percentage of mannosylated lipid within the liposome increased the size of the resulting particles decreased (Table 1). This holds true for both mono- and tri-mannose substituted phospholipids. This decrease in particle size can be attributed to a more conical packing parameter of mannose-conjugated phospholipids (due to the large carbohydrate head group) compared with the more cylindrical packing parameter of the phosphatidylcholine phospholipid. As more mannose-conjugated phospholipid is incorporated into the liposome bilayer, the angle of curvature of the liposomal membrane is increased, resulting in progressively smaller particles.

Entrapment of FITC-ovalbumin within the liposomes was consistent across all formulations with no significant variation relative to the degree of mannosylation of the formulations ($P > 0.05$) (Table 1). Similarly, stability of the liposome membrane was not affected greatly by increasing the percentage of mannosylated lipid within the bilayer as FITC-ovalbumin was retained equally well over 14 days by most of the liposome formulations. The formulations containing 10% and 20% mannosylated phospholipid lost a small percentage of fluorescent protein over the period studied but entrapment was still high in all cases (≥ 88%), regardless of the percentage of mannosylated lipid incorporated. Thus, while a high degree of mannosylation does appear to impair the stability of the liposome bilayer somewhat, a significant proportion of the initial protein load is still available to be delivered to the dendritic cell.

To assess the presence of mannose residues on the surface of the liposomes, Con A was added to the dispersions to a final concentration of $8\,\mu g\,mL^{-1}$. Con A is a carbohydrate-binding lectin and will bind to accessible mannose molecules resulting in an increase in the mean particle size. Addition of Con A to liposome dispersions resulted in an increase in mean particle size in formulations containing mannosylated lipid (Figure 1). The increase in mean particle size was proportional to the amount of

Table 1 Characteristics of liposome formulations

Percentage of mannosylated lipid (w/w)	Monomannose			Trimannose		
	Size[a] (nm)	Entrapped protein[b] ($mg\,mL^{-1}$)	% Protein retained after 14 days	Size[a] (nm)	Entrapped protein[b] ($mg\,mL^{-1}$)	% Protein retained after 14 days
0	987 ± 100	0.65	100	1090 ± 46	0.54	100
1	964 ± 53	0.68	100	928 ± 66	0.55	100
5	794 ± 61	0.63	100	841 ± 52	0.53	100
10	683 ± 77	0.59	94	599 ± 23	0.60	97
20	627 ± 14	0.54	97	658 ± 19	0.56	88

[a]Values represent the mean ± s.d. of ten measurements. [b]Values represent the mean of four separate experiments determined on the day of liposome preparation. Differences in protein entrapment between formulations are not significant ($P > 0.05$).

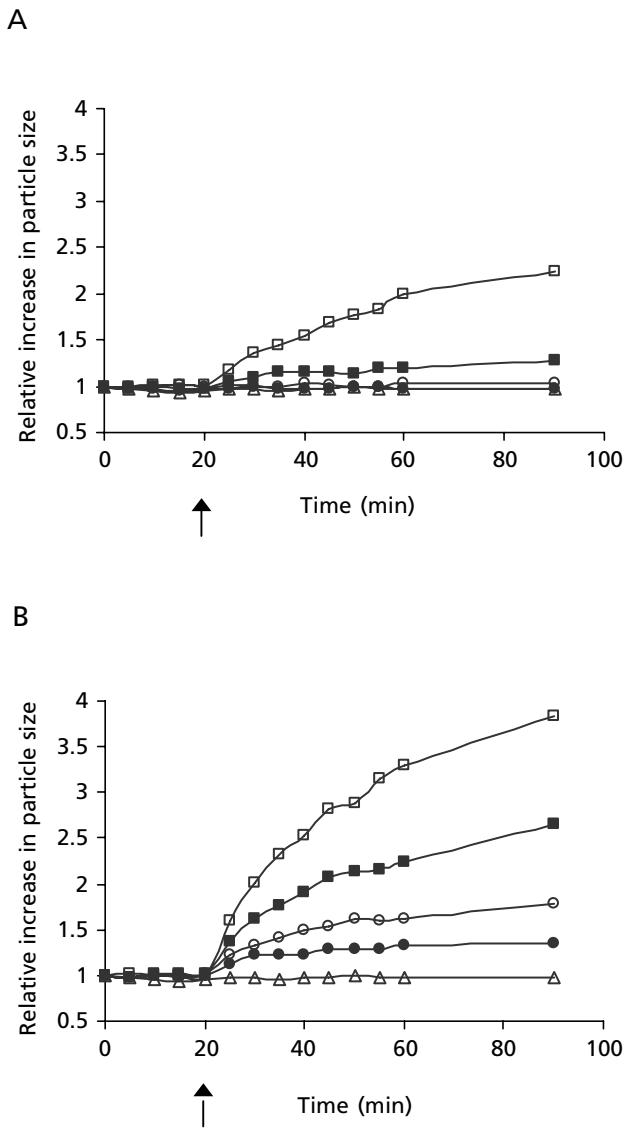

Figure 1 Relative increase in mean particle size of liposome preparations containing M_1-DPPE (A) or M_3-DPPE (B) after addition of Con A. Particles size measurements of formulations containing 0% mannosylated phospholipid (Δ), 1% mannosylated phospholipid (●), 5% mannosylated phospholipid (○), 10% mannosylated phospholipid (■) and 20% mannosylated phospholipid (□) were made by photon correlation spectroscopy at 5-min intervals. Con A was added to a final concentration of $8\,\mu mL^{-1}$ at t = 20 min as indicated by the arrow, and measurements continued.

mannosylated lipid incorporated into the formulation, with the liposomes composed of 20% mannosylated lipid showing the largest increase in mean particle size and formulations containing 0% mannosylated lipid showing no increase in mean particle size (Figure 1). The increase in particle size after addition of Con A was also more pronounced in particles containing the branched tri-mannosylated lipid compared with mono-mannose-containing liposomes, reflecting multiple binding sites for the lectin on the surface of the liposomes. These results indicate that the use of mannose-conjugated phospholipids is an effective means of attaching mannose residues to the surface of liposomes.

Uptake of liposomes by dendritic cells

Uptake of mannosylated liposomes by dendritic cells was determined by incubating MoDCs and BMDCs with liposomes containing the model antigen FITC-ovalbumin for 48 h then measuring the resulting cell-associated fluorescence by flow cytometry. Uptake of mono-mannose-containing formulations by $CD1a^+$ MoDCs (Figure 2A) was not affected by including mannosylated phospholipids in the liposome bilayer. None of the formulations containing mono-mannose showed enhanced uptake compared with the non-mannosylated formulation. For the tri-mannose-containing formulations, there was an increase in uptake of formulations containing tri-mannosylated lipid within the liposome bilayer (Figure 2B) compared with non-mannosylated liposomes. However, only the 20% tri-mannose-containing formulation showed a significant increase in uptake over non-mannosylated liposomes ($P < 0.05$). The lack of statistical significance may in part be explained by the large interindividual variation found between peripheral blood donors. The trend towards increasing uptake with increasing M_3-DPPE was, however, consistent across experiments and confirms that targeting dendritic cells via the mannose receptor is a viable option for increasing antigen uptake by these cells. The lack of increased uptake with the mono-mannosylated formulations by MoDCs may reflect the complexity of mannose-binding receptors on dendritic cells and their preference for multivalent ligands (Kery et al 1992; Taylor& Drickamer 1993; Stahl & Ezekowitz 1998).

Investigation into the uptake of mono-mannose-containing liposomes by $CD11c^+$ BMDCs showed a small, but non-significant, increase in uptake with increasing degree of mannosylation (Figure 2C). For the tri-mannose-containing formulations, however, no such trend was evident (Figure 2D). There appears to be no increase in uptake of tri-mannose-containing formulations by BMDCs with increasing degree of mannosylation. In fact, the liposomes containing the highest percentage of tri-mannosylated lipid (20% M_3-DPPE) showed the only significantly different level of uptake compared with non-mannosylated liposomes, and this was a decrease in uptake. While BMDCs are known to have receptors on their surface able to recognise and bind carbohydrates (Stahl et al 1980; Garcon et al 1988), the lack of enhanced uptake compared with human MoDCs may reflect the interspecies differences in receptor specificity. To the best of our knowledge, only one other study has directly compared the interaction of mannosylated liposomes with human MoDCs and murine BMDCs (Foged et al 2004). In their study, Foged et al found that while mannosylated liposomes were able to interact with both dendritic cell types to a greater extent than non-mannosylated liposomes, only the interaction with MoDCs could be blocked using mannan. In our experiments we could not demonstrate enhanced interaction of mannosylated liposomes with BMDCs but these

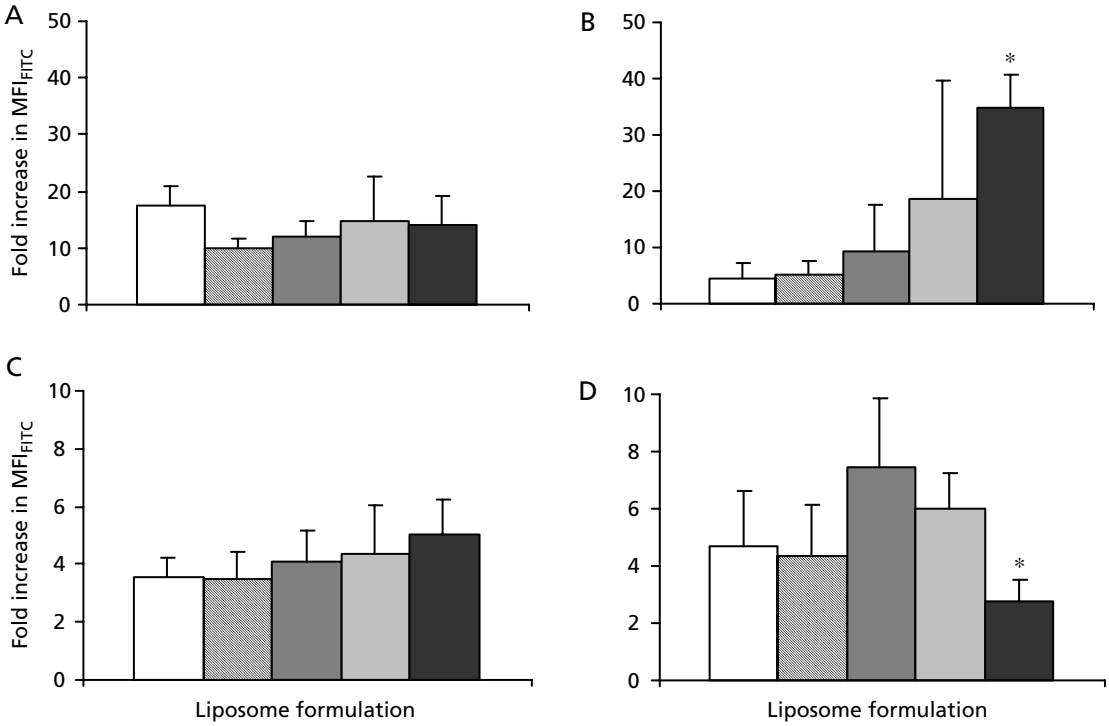

Figure 2 Uptake of liposomes by MoDCs (human monocyte-derived dendritic cells) (A and B) and BMDC (murine bone-marrow-derived dendritic cells) (C and D). Uptake of M_1-DPPE (A and C) or M_3-DPPE (B and D) liposomes containing 0% mannosylated lipid (□), 1% mannosylated lipid (■)5% mannosylated lipid (■), 10% mannosylated lipid (■) and 20% mannosylated lipid (■) was determined by comparing the mean fluorescence intensity of dendritic cells pulsed with FITC-ovalbumin containing liposomes (MFI_{FITC}) with that of unpulsed dendritic cells and is presented as a fold increase in MFI_{FITC}. Bars represent the mean + s.d. of three experiments. $^*P < 0.05$, compared with 0% mannosylated phospholipid formulations.

studies suggest that differences in binding affinity of carbohydrate-binding receptors do exist between dendritic cells cultured from different mammalian species.

Dendritic cell activation

Dendritic cell activation was determined by measuring the level of expression of activation markers on $CD1a^+$ MoDCs and $CD11c^+$ BMDCs pulsed with mannosylated liposomes and comparing these levels with those expressed on dendritic cells not pulsed with liposomes (Table 2). Neither mono- nor tri-mannosylated liposome formulations showed any enhanced ability to activate MoDCs compared with the non-mannosylated liposomes, consistent with earlier findings in our laboratory (Copland et al 2003). In fact, all of the mono-mannosylated liposomes resulted in a small, but significant, reduction in the expression of both CD80 and CD86 markers on MoDCs compared with non-mannosylated liposomes. In addition, none of the liposome formulations were able to stimulate up-regulation of either CD80 or CD86 on the surface of MoDCs to the same extent as the LPS positive control. It is somewhat surprising that the increased uptake of tri-mannosylated liposomes into MoDCs did not result in an increase in activation marker expression on the surface of

Table 2 Fold increase in activation marker expression on dendritic cells after incubation with M_1-DPPE- and M_3-DPPE-containing liposomes for 48 h compared with activation marker expression on dendritic cells not pulsed with liposomes

	MoDCs		**BMDCs**	
	CD80	CD86	CD86	CD40
0% Mannose	2.18 ± 0.03	2.1 ± 0.4	1.9 ± 0.3	1.8 ± 0.3
% M_1-DPPE				
1%	1.6 ± 0.1*	1.5 ± 0.3*	1.3 ± 0.1	1.4 ± 0.3
5%	1.43 ± 0.03*	1.4 ± 0.2*	1.4 ± 0.1	1.5 ± 0.2
10%	1.35 ± 0.08*	1.4 ± 0.2*	1.3 ± 0.1	1.4 ± 0.2
20%	1.35 ± 0.05*	1.41 ± 0.02*	1.5 ± 0.3	1.6 ± 0.5
% M_3-DPPE				
1%	1.9 ± 0.4	1.8 ± 0.6	2.4 ± 0.8	1.9 ± 0.2
5%	2.0 ± 0.8	2.2 ± 0.9	4.1 ± 1.8	2.6 ± 0.6
10%	1.7 ± 0.2	2.0 ± 0.3	4.0 ± 1.5	2.5 ± 0.8
20%	1.6 ± 0.4	1.9 ± 0.1	2.4 ± 0.8	2.0 ± 0.6

MoDCs, human monocyte-derived dendritic cells; BMDCs, murine bone-marrow-derived dendritic cells. Data represents the mean ± s.d. of three independent experiments. $^*P < 0.05$, compared with activation marker expression on MoDCs pulsed with the 0% mannose liposome formulation.

the dendritic cells. However, unlike the ligation of TLRs, which triggers a well-characterised intracellular signalling cascade resulting in the up-regulation of co-stimulatory molecules on the surface of dendritic cells and increased cytokine production (Kaisho & Akira 2001), the intracellular signalling occurring downstream of C-type lectin ligation is less well characterised. Ligation of the MR has been linked to an increase in cytokine production but the mechanism for this increase is unclear (Fraser et al 1998). Targeting of mannosylated liposomes to C-type lectins on dendritic cells in the absence of other danger signals may therefore not be sufficient to stimulate dendritic cell activation.

The apparent decrease in MoDC activation after incubation with mannosylated formulations compared with non-mannosylated formulations is also intriguing. Recent evidence in the literature suggests that ligation of C-type lectins may in fact lead to a down-regulation of the immune response (Nigou et al 2001; Geijtenbeek et al 2003) and is a possible mechanism of persistence of mycobacteria in chronic infections. It is possible that the mannosylated ligands used in these experiments also stimulated a down-regulation of the immune response.

The expression of murine dendritic cell activation markers CD86 and CD40 on $CD11c^+$ BMDCs was similarly determined. Expression of both CD86 and CD40 appeared to be slightly higher after incubation with tri-mannose-containing liposomes compared with mono-mannose containing liposomes but in general none of the mannosylated liposome formulations resulted in significantly higher levels of BMDC activation when compared with non-mannosylated liposomes and no liposome formulation was able to activate BMDCs to the same extent as LPS (positive control).

T cell proliferation

Proliferation of $V\alpha2^+$ $CD8^+$ splenocytes from OT-I transgenic mice was investigated after incubation with BMDCs pulsed with mannosylated liposomes containing ovalbumin protein as a model antigen. While there was an increase in T cell proliferation in cultures incubated with BMDCs pulsed with liposomes containing increasing amounts of mono-mannosylated phospholipid (Figure3A), these differences were not statistically significant. It also appeared that BMDCs pulsed with tri-mannosylated liposomes (Figure3B) stimulated greater proliferation of $CD8^+$ T cells compared with BMDCs pulsed with mono-mannosylated liposomes (Figure 3A). However, none of the mannosylated liposome formulations were significantly different from the non-mannosylated liposome formulations studied. This lack of T cell stimulation is not surprising considering the lack of dendritic cell activation after incubation with the liposome formulations. Internalisation of antigen by dendritic cells is not enough to guarantee an effective T cell response. The dendritic cells must be able to process and present the antigen on the cell surface along with co-stimulatory molecules to ensure efficient activation of the naïve T cells.

We also investigated the ability of BMDCs pulsed with mannosylated liposomes to stimulate proliferation of $CD4^+$ T cells from OT-II transgenic mice and found no proliferation of $CD4^+$ T cells after incubation with BMDC exposed to any of the formulations used (data not shown).

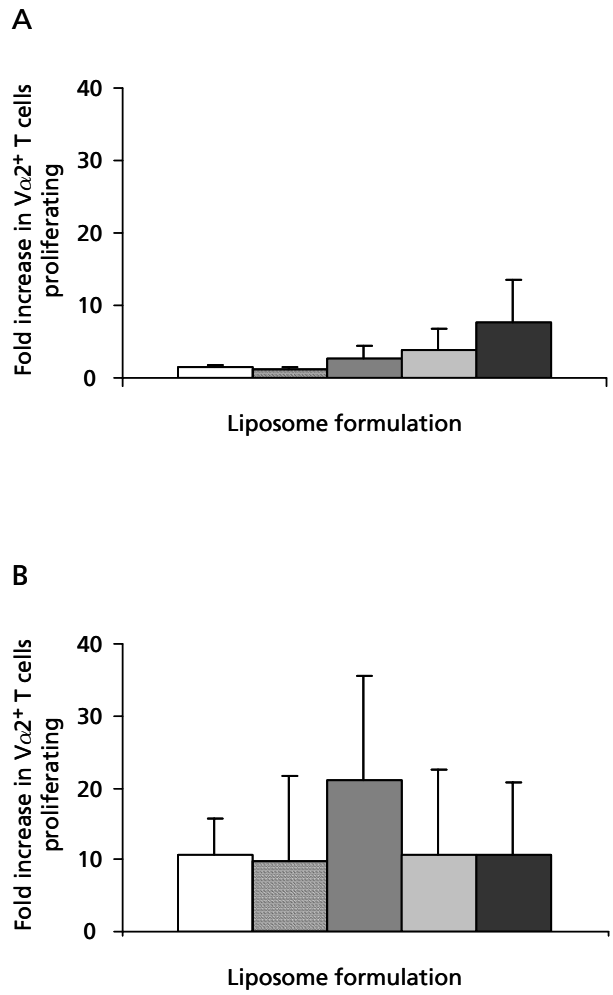

Figure 3 Increase in the percentage of $V\alpha2^+$ OT-I splenocytes proliferating after incubation with BMDCs (murine bone-marrow-derived dendritic cells) pulsed with liposome formulations. CFSE (carboxyfluorescein diacetate succinimidyl ester)-labelled splenocytes from an OT-I transgenic mouse were incubated for 72 h with BMDCs pulsed with M_1-DPPE (A) or M_3-DPPE (B) liposomes containing 0% mannosylated lipid (□), 1% mannosylated lipid (▨), 5% mannosylated lipid (▩), 10% mannosylated lipid (■) and 20% mannosylated lipid (■). Cells were stained with the T cell marker Vα2 then proliferation of $V\alpha2^+$ cells determined by flow cytometry. The level of proliferation in splenocytes incubated with unpulsed BMDC was also determined. Data is presented as the fold increase $V\alpha2^+$ cells proliferating in cultures incubated with liposome-pulsed BMDCs compared with unpulsed BMDCs. Bars represent the mean + s.d. of three experiments.

Conclusions

Previous studies have investigated targeting antigens to dendritic cells using mannosylated liposomes as a delivery system (Garcon et al 1988; Sugimoto et al 1995;

Foged et al 2004). We have previously demonstrated that including a branched tri-mannose phospholipid in the liposome bilayer is an effective means of increasing the uptake of a model antigen by MoDCs, and increasing proliferation of primed T cells in-vitro (Copland et al 2003). In this study we investigated the effects on immunogenicity of varying the amount of mannosylated phospholipid incorporated into liposomes, and also the effect of using a branched tri-mannose-containing phospholipid compared with a mono-mannose-containing phospholipid as the targeting moiety. We also compared the immunogenicity of the different liposome preparations in both human MoDCs and murine BMDCs. We have found important differences between the two dendritic cell types in terms of their ability to take up a model antigen contained inside mannosylated liposomes. Human MoDCs were able to take up liposomes displaying the more complex tri-mannose ligand on the liposome surface to a much greater extent than liposomes displaying a simple mono-mannose ligand, even when 20% mono-mannosylated phospholipid was incorporated into the liposomes. Murine BMDCs, however, were not able to take up liposomes displaying either ligand on the liposome surface to a greater degree than non-targeted liposomes. This may reflect either a lack of mannose-binding receptor expression on the surface of BMDCs or else differences in receptor affinity for the ligands used in this study. Despite the increased uptake of mannosylated liposomes by MoDCs, this did not translate to an increase in dendritic cell activation. Neither were BMDCs pulsed with mannosylated liposomes able to stimulate enhanced $CD8^+$ T cell proliferation compared with BMDCs pulsed with non-targeted liposomes. These results suggest that important differences do exist in terms of receptor expression and ligand affinity for C-type lectins on dendritic cells. It also confirms previous reports that targeting of antigens to C-type lectins does not necessarily result in enhanced dendritic cell activation or subsequent immune responses.

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