Mannan-mediated gene delivery for cancer immunotherapy

Choon K. Tang,[1] Jodie Lodding,[1] Gabriela Minigo,[2] Dodie S. Pouniotis,[1] Magdalena Plebanski,[2] Anja Scholzen,[2] Ian F. C. McKenzie,[1] Geoffrey A. Pietersz[3]* and Vasso Apostolopoulos[1]*

[1]*Immunology and Vaccine Laboratory,* [2]*Vaccine and Infectious Diseases Laboratory, and* [3]*Bio-Organic and Medicinal Chemistry Laboratory, Burnet Institute at Austin, Heidelberg, VIC, Australia*

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Correspondence: Professor Vasso Apostolopoulos, Burnet Institute at Austin, Kronheimer Building, Studley Road, Heidelberg, VIC 3084, Australia.
Email: vasso@burnet.edu.au
Senior author: Vasso Apostolopoulos
*V. Apostolopoulos and G. A. Pietersz contributed equally to this work.

Summary

Recent years have seen a resurgence in interest in the development of efficient non-viral delivery systems for DNA vaccines and gene therapy. We have previously used oxidized and reduced mannan as carriers for protein delivery to antigen-presenting cells by targeting the receptors that bind mannose, resulting in efficient induction of cellular responses. In the present study, oxidized mannan and reduced mannan were used as receptor-mediated gene transfer ligands for cancer immunotherapy. *In vivo* studies in C57BL/6 mice showed that injection of DNA encoding ovalbumin (OVA) complexed to oxidized or reduced mannan-poly-L-lysine induced CD8 and CD4 T-cell responses as well as antibody responses leading to protection of mice from OVA+ tumours. Both oxidized and reduced mannan delivery was superior to DNA alone or DNA-poly-L-lysine. These studies demonstrate the potential of oxidized and reduced mannan for efficient receptor-mediated gene delivery *in vivo*, particularly as DNA vaccines for cancer immunotherapy.

Keywords: DNA vaccine; mannan; mannose receptor; gene therapy; cancer; oxidized mannan

Introduction

Since the emergence of DNA vaccines in the early 1990s, many studies have been performed to increase their efficacy in humans. One such strategy involves the use of viral or non-viral carriers to ensure the entry of the DNA into cells. The use of viral vectors is limited by the size of the genetic material they can deliver, their immunogenicity and their oncogenic potential. In the light of these concerns, non-viral gene delivery has emerged as a promising alternative. Non-viral vectors include lipids,[1] synthetic polymers,[2] peptides[3] and microparticles.[4] These vectors have the advantages of low cost and immunogenicity. However, their use is still limited by a relatively low transfection efficiency compared with viral vectors.

Receptor-mediated gene targeting has been used to increase the efficiency of DNA incorporation into specific target cell types, particularly for gene therapy. Several receptors have been targeted, including the mannose receptor for macrophage targeting,[5] asialoglycoprotein for hepatocyte targeting,[6] and neurotensin for central nervous system cell targeting.[6–10] In particular, the mannose receptor has been targeted using DNA complexed to mannosylated poly L-lysine (PLL), polyethyleneimine (PEI) and liposomes.[5,11,12] The mannose receptor is expressed on macrophages and dendritic cells (DCs) and is a preferable target for introducing genetic material encoding tumour antigens as a DNA vaccine.

We previously demonstrated that mannan (poly-mannose) conjugated to Mucin 1 (MUC1; an antigen found on adenocarcinoma cells) fusion protein in the oxidized form (OxMan; comprising aldehydes) or in the reduced form (RedMan; comprising aldehydes reduced to alcohols) generated differential T helper type 1 Th1/Th2 immune

Abbreviations: CD4, OVA323-339 major histocompatibility complex (MHC) class II epitope from ovalbumin; CD8, MHC class I epitope from ovalbumin SIINFEKL; Con A, concanavalin A; DC, dendritic cell; OVA, ovalbumin; OxMan, oxidized mannan; PAS, periodic acid Schiff's stain; PLL, poly L-lysine; RedMan, reduced mannan; *R* value, DNA (PO_4^-):PLL (NH_3^+) molar ratio.

responses.[13–17] The OxMan conjugate was found to be endocytosed by the mannose receptor and allowed fast tracking from the endosome into the cytoplasm as a result of the aldehyde residues present.[18] One of the major rate-limiting and inefficient steps in DNA vaccination is the escape of DNA from the endosomes into the cytosol. Failure of DNA to traverse the endosomal membrane results in translocation into the lysosomal compartment and its degradation, with inefficient transgene expression.

In this study, we investigated the *in vivo* efficacy of OxMan and RedMan as receptor-mediated gene transfer ligands. Using a poly cationic linker, PLL, to link OxMan and RedMan to DNA, we demonstrated successful immune responses (CD8, CD4 and antibody responses) leading to tumour protection in mice.

Materials and methods

Preparation of oxidized and reduced mannan-PLL

To prepare oxidized mannan (OxMan), 14 mg of mannan (from *Saccharomyces cerevisiae*; Sigma, St Louis, MO) was dissolved in 1 ml of sodium phosphate buffer (pH 6.0, 0.1 M) to which 100 μl of 0.1 M sodium periodate (dissolved in phosphate buffer, pH 6) was then added, and the mixture was incubated on ice for 1 hr in the dark. Ten microlitres of ethanediol was added to the mixture which was incubated for a further 30 min on ice. The mixture was passed through a PD-10 column (pre-equilibrated in 0.01 M phosphate buffer, pH 8; Amersham Bioscience, Uppsala, Sweden) and oxidized mannan was collected in the first 2 ml after the void volume (2.5 ml). One milligram of PLL [average chain length 212; relative molecular mass (M_r) 27 400; Sigma, Steinheim, Germany] dissolved in phosphate-buffered saline (PBS) was added to 2 ml of OxMan. The oxidized mannan-PLL (OxMan-PLL) mixture was allowed to react in the dark at room temperature (RT) overnight. Finally, OxMan-PLL was dialysed against 5 mM NaCl for 24 hr. Reduced mannan-PLL (RedMan-PLL) was prepared by adding 1 mg of sodium borohydride to the OxMan-PLL conjugate and incubating the mixture for 3 hr at RT before dialysis. The final concentrations of mannan and PLL in both OxMan-PLL and RedMan-PLL were 7 and 0.5 mg/ml, respectively.

DNA plasmid preparation

The plasmid sOVA-C1, incorporating the cytomegalovirus promoter, which expresses ovalbumin, was kindly provided by Dr Peter Smooker (RMIT University, Melbourne, Australia)[19] (referred to as 'OVA DNA' hereafter). DNA plasmids were purified using the Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. OVA DNA used in gel retardation experiments was linearized by *Eco*RI digestion. The quality of OVA DNA was tested by restriction enzyme digestion on agarose gels, transfection using fugene into CHO-K1 cells, and determination of OVA expression by flow cytometry (data not shown).

Complexation efficiency of carrier-PLL, PLL and plasmid DNA

Both OxMan-PLL and RedMan-PLL were complexed to plasmid DNA using the same method. OxMan-PLL-DNA and RedMan-PLL-DNA conjugates were complexed at various DNA (PO_4^-):PLL (NH_3^+) molar ratios (abbreviated as *R*). Plasmid DNA in various amounts (μg) was dissolved in 0.7 M sodium chloride. To this, an equal volume of OxMan-PLL (in quantities to give *R* values of 0, 0.25, 0.5, 0.75 and 1) in the same final NaCl concentration was added in a stepwise manner (10 ml per addition) for 1 hr. The conjugates, OxMan-PLL-DNA and RedMan-PLL-DNA, were incubated at RT for 30 min before use. The degree of complexation between OxMan-PLL and DNA under different conditions, i.e. different concentrations and *R* values, was determined from the extent of DNA retardation in 0.6% (w/v) agarose gel electrophoresis run for 1 hr at 100 mV. The amount of conjugate in micrograms refers to the amount of plasmid DNA unless otherwise stated.

Cytotoxicity of carriers

J774 cells (1×10^5) in a volume of 150 ml of medium were seeded into each well of a 96-well microtitre plate. A volume of 50 ml of OxMan, Redman, OxMan-PLL, RedMan-PLL and PLL at various concentrations was incubated with the cells for 16 hr at 37° and subsequently 1 μCi of thymidine was added and the mixture was incubated for a further 6 hr. Cells were harvested and [^{3}H]thymidine uptake was measured using a β-scintillation counter (Top Count Gamma Counter; Packard, Meriden, CT).

Mice and immunizations

Female C57BL/6 mice aged 6–10 weeks were used in all experiments. Mice were immunized twice intradermally (i.d.) into the base of the tail with 100 μl of the following: DNA 10 μg, DNA 50 μg, DNA-PLL 10 μg, DNA-PLL 50 μg, RedMan-PLL-DNA 10 μg, RedMan-PLL-DNA 50 μg, OxMan-PLL-DNA 10 μg or OxMan-PLL-DNA 50 μg. Spleens were removed 10–14 days after the last immunization and immune responses were assessed. All studies were reviewed and approved by the Austin Health animal ethics committee.

Antigens

For *in vitro* stimulation, the following antigens were used: chicken egg OVA (Sigma, Steinheim, Germany), OVA

CD4 epitope ISQAVHAAHAEINEAGR (OVA323-339) and OVA CD8 epitope SIINFEKL. All peptides were synthesized by Mimotopes (VIC, Clayton, Australia) and were >95% pure by mass spectrometry and high-performance liquid chromatography (HPLC).

Proliferation assay

Splenocytes (2×10^5) in 100 μl of complete medium [RPMI supplemented with 10% fetal calf serum (FCS), penicillin/streptomycin and glutamine] were seeded into 96-well round-bottom plates and cultured in triplicate with 10 μg/ml OVA, OVA CD8 epitope, OVA CD4 epitope or medium alone (negative control) and incubated at 37° and 5% CO_2. Concanavalin A (Con A) at a concentration of 1 μg/ml was used as an internal positive control. Proliferation was assessed on days 2–5 by adding 1 μCi of [^{3}H]thymidine per well to one plate per time-point. Cells were incubated for a further 6 hr before harvesting onto glass fibre filters. [^{3}H] uptake was measured using a β-scintillation counter (Top Count Gamma Counter).

Enzyme-linked immunosorbent spot-forming cell assay (ELISPOT)

Ninety-six-well plates (MAIP S4510; Millipore, Moisheim, France) were pre-wet with 50 μl of 70% ethanol, washed six times with 200 μl of sterile PBS and coated with 70 μl of 5 μg/ml anti-interferon (IFN)-γ coating antibody, AN18 (Mabtech, Stockholm, Sweden), overnight at 4°. Plates were washed six times with sterile PBS and blocked with 200 μl of complete medium for 2 hr at 37°. Spleen cells (5×10^5) in 100 μl of complete medium were incubated with 10 μg/ml OVA, 10 μg/ml OVA CD8 epitope, 10 μg/ml OVA CD4 epitope or medium alone for 18 hr at 37°. Con A at a concentration of 1 μg/ml was used as an internal positive control. Triplicate wells were set up for each condition. Plates were developed by incubation with the biotinylated anti-IFN-γ antibody R46A2 (Mabtech) for 2 hr at RT followed by incubation with 1 μg/ml streptavidin-alkaline phosphatase (ALP) (Mabtech) for 30 min at RT. Spots of activity were detected using a colorimetric AP kit (Bio-Rad, Hercules, CA) and counted using an AID ELISPOT reader (Autoimmun Diagnastika GmbH, Strassberg, Germany). Data are presented as mean spot forming units (s.f.u.) per 5×10^5 cells ± standard error of the mean (SEM).

Enzyme-linked immunosorbent assay (ELISA)

Serum was collected from mice before and 2 weeks after final immunization. Polyvinyl chloride microtitre plates were coated with OVA [10 mg/ml in coating buffer (0.05 M sodium bicarbonate, pH 9.6)] overnight at 4°. Plates were blocked with 2% bovine serum albumin (BSA)/PBS for 2 hr at 37° in a humidified box. Plates were washed with PBS/0.05% Tween-20 five times and incubated for 2 hr at RT with mouse sera from immunized mice at various dilutions, and then washed 15 times and incubated with horseradish-peroxidase-conjugated sheep anti-mouse immunoglobulin (Ig) (Amersham Bioscience, Buckinghamshire, UK) for 2 hr at RT. After the plates had been washed, developing buffer [5 ml of ABTS buffer (1 M sodium hydrogen phosphate and 1 M citric acid, pH 4.5), 100 ml of ABTS stock and 4 ml of H_2O_2] was added and the mixture was incubated for 15–30 min. Plates were read on a Fluostar Optima microplate reader (BMG Labtech, Offenburg, Germany) at 405 nm.

Tumour studies

Groups of female C57BL/6 mice (six mice/group) were immunized with 10 μg ($R = 0.4$) and 50 μg ($R = 2$) of OVA DNA complexed to PLL, RedMan-PLL or OxMan-PLL at [NaCl] = 0.7 M. Mice were injected twice on days 0 and 14 intradermally at the base of the tail. Ten days after the final injection, mice were challenged with 1×10^7 EG7 tumour cells (OVA-transfected EL4 cells) in 100 μl of PBS subcutaneously. EL4 tumours were included as a specificity control. Tumours were measured every second day.

Statistical analysis

Mean values were compared using Student's two-tailed *t*-test. A *P*-value threshold of $P < 0.05$ indicates a statistically significant difference. Significant differences among multiple groups in tumour challenge experiments were analysed by one-way analysis of variance (ANOVA) and the Tukey post hoc test using GraphPad prism 4.0 software (GraphPad Software, San Diego, CA).

Results

Efficiency of conjugation between OxMan and PLL

DNA cannot be directly conjugated to OxMan or RedMan. To facilitate binding of DNA, OxMan-PLL or RedMan-PLL conjugates were used as described in the Materials and methods. The binding interaction between OxMan and PLL is via covalent bonds, while the complexation of DNA to OxMan-PLL is by an electrostatic interaction between the negatively charged phosphate group (PO_4^-) of DNA and the positively charged amino group (NH_3^+) present in lysine. To confirm binding between OxMan and PLL, the carrier complex (OxMan-PLL) was analysed on a sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) gel stained with Coomassie blue or the carbohydrate stain periodic acid Schiff's (PAS). OxMan showed no staining with Coomassie blue, as expected (Fig. 1a, lane A). Coomassie

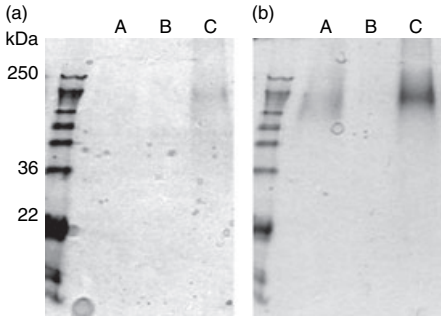

Figure 1. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) gel. (a) Coomassie blue stain and (b) periodic acid Schiff's (PAS) stain. Lane A, oxidized mannan (OxMan); lane B, poly L-lysine (PLL) and lane C, OxMan-PLL.

blue did not stain PLL (Fig. 1a, lane B) because of the hydrophilic nature of PLL, which resists forming micelles with the hydrophobic SDS which are necessary for it to migrate in the gel. However, OxMan-PLL was stained (Fig. 1a, lane C), demonstrating that PLL was conjugated to OxMan. PAS stained both OxMan and OxMan-PLL, thus demonstrating that there was conjugation between OxMan and PLL (Fig. 1b, lanes A and C).

Characterization of OxMan-PLL and OVA DNA complexes

The interaction of DNA with OxMan-PLL was analysed using agarose gel electrophoresis. Upon complexation between OxMan-PLL and DNA, the DNA loses its negative charge and consequently its ability to migrate in an agarose gel. A major factor for consideration in the complexation between polyamines and DNA is the molar ratio of PO_4^- groups present in DNA and NH_3^+ groups present in the polyamines (R value). The degree of complexation between OxMan-PLL and OVA DNA was assessed at various R values (0.1–10) (Fig. 2a). The degree of retardation decreased as R increased, indicating weaker DNA binding at increased PO_4^-:NH_3^+ ratios. At $R = 0.1$, retardation of DNA was complete, whereas at $R = 3$–10 no retardation of DNA in OxMan-PLL complexes was observed. On the basis of these results, we chose $R = 0.4$ (mostly retarded) and $R = 2$ (little retardation) for further study. It has been reported that, for interaction of free PLL and mannose-PLL with DNA, the molar concentration of salt (NaCl) in the complexation mixture affects the degree of complexation.[20] Thus, we used 0.7 M NaCl for complexation of the OxMan-PLL and RedMan-PLL conjugates with OVA DNA.[20] DNA mixed with OxMan showed no binding (Fig. 2b), whilst PLL completely bound DNA at all R values (Fig. 2c). Hence, OxMan alone did not affect the migration of DNA and OxMan-PLL was required for complexation of DNA. Similar interactions were demonstrated for RedMan-PLL conjugates and DNA (data not shown).

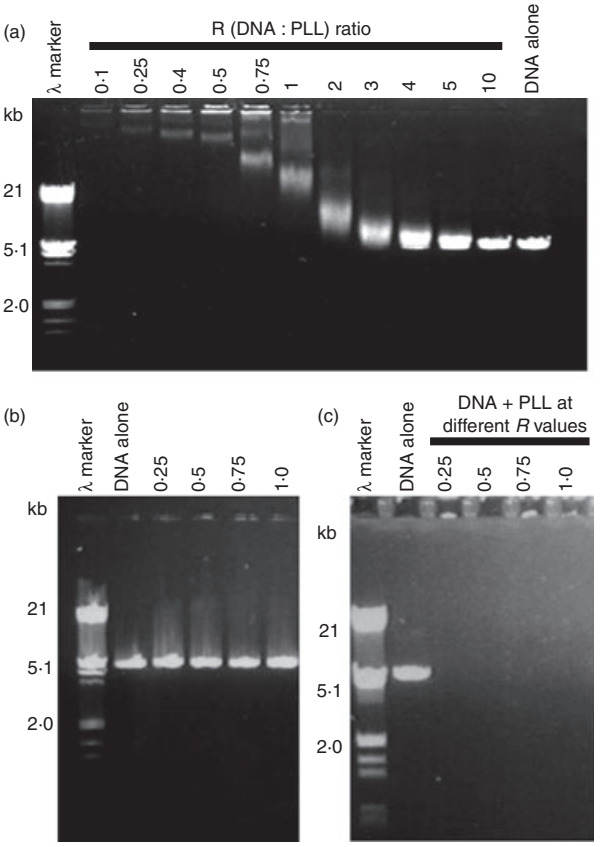

Figure 2. (a) Degree of complexation between oxidized mannan–poly L-lysine (OxMan-PLL) and ovalbumin (OVA) DNA at different PLL:DNA ratios (R). OxMan-PLL-DNA complexes of R values 0.1, 0.25, 0.4, 0.5, 0.75, 1, 2, 3, 4, 5 and 10 were used to evaluate the degree of complexation by assessing the extent of gel retardation in 0.6% agarose gel. Varying levels of complexation between the two components were observed at different R values, as reflected by the extent of retardation of bands. The degree of complexation decreased as the R value increased; 10 times excesses of PLL and DNA showed complete and no binding, respectively. The amount of DNA loaded into wells for each condition was the same (200 ng). (b) Gel retardation analysis for DNA and OxMan. DNA mixed with OxMan did not show signs of retardation (migration to the anode) and showed the same bands as DNA alone at all R values. (c) Gel retardation analysis for DNA and PLL. For PLL mixed with DNA, all groups showed complete retardation.

OxMan and RedMan protect cells from the cytotoxic effect of PLL

Polycations are cytotoxic to cells, so in order to establish the level of cytotoxicity to cells, PLL, OxMan, RedMan, OxMan-PLL and RedMan-PLL were incubated with J774 cells and cell viability was determined by measuring [^{3}H]thymidine incorporation (Fig. 3). PLL alone added to J774 cells had an IC_{50} (concentration at 50% inhibition) of 18 μg/ml, while OxMan and RedMan had IC_{50}s of >125 μg/ml. OxMan-PLL and RedMan-PLL had IC_{50}s of 60 and >120 μg/ml, respectively. Thus, conjugation of

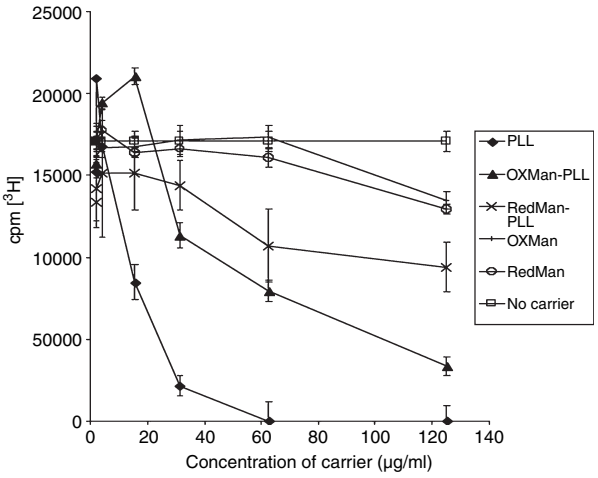

Figure 3. Cell toxicity assay. Poly L-lysine (PLL), oxidized mannan–poly L-lysine (OxMan-PLL), reduced mannan–poly L-lysine (RedMan-PLL), OxMan, RedMan or no carrier was added to J774 cells. [^{3}H]thymidine was added after 16 hr and thymidine uptake by cells was measured. Error bars depict standard error of the mean for triplicate wells. A representative example of five different experiments is shown.

OxMan or RedMan to PLL reduced the cytotoxic effect of PLL 4-fold or 8-fold, respectively. This was also confirmed by propidium iodide (PI; Sigma, Steinheim, Germany) staining of cells as detected by flow cytometry (data not shown). Furthermore, we also showed that the percentage inhibition of [^{3}H] uptake correlated with cell viability as measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (data not shown).

Immune responses to OxMan and RedMan-PLL-OVA DNA in C57BL/6 mice

OVA DNA complexed to OxMan-PLL or RedMan-PLL was used to immunize C57BL/6 mice. Two complexes with $R = 0.4$ and $R = 2.0$ were chosen for *in vivo* immune responses and efficacy studies based on complete and partial DNA complexation to OxMan-PLL and RedMan-PLL (Fig. 2). To keep the amounts of OxMan and RedMan in the $R = 0.4$ and $R = 2.0$ conjugates the same (150 μg), two doses (10 and 50 μg) were chosen for immunization of mice. Groups of four mice were given two injections of 10 μg ($R = 0.4$) or 50 μg ($R = 2.0$) of either OxMan-PLL-OVA DNA or RedMan-PLL-OVA DNA on days 0 and 14. DNA alone and PLL-DNA were used as controls.

Proliferation assay

Proliferation assays were used to determine the level of antigen-specific T cells by measuring the [^{3}H]thymidine uptake of T cells proliferating in the presence of peptides on days 1–5. There was no proliferation of T cells from mice immunized with DNA alone (10 or 50 μg) or PLL-DNA (10 or 50 μg) (Fig. 4a–d). Mice immunized with RedMan-PLL-DNA (10 μg of DNA in the RedMan-PLL-DNA complex) generated T cells, which proliferated primarily in response to the CD4 OVA T-cell epitope and OVA (Fig. 4e), although low levels of T cells responding to the CD8 epitope were generated. However, at a higher immunization dose (50 μg of DNA in the RedMan-PLL-DNA complex), T cells recognized both the CD4 and the CD8 OVA T-cell epitopes (Fig. 4f); there was also a reaction to OVA. There was a significant difference ($P < 0.05$) in CD8 T-cell responses between the 10 and 50 μg DNA doses of RedMan-PLL-DNA on days 2 and 3. Mice immunized with OxMan-PLL-DNA generated T cells that recognized only the CD8 T-cell epitope and OVA at the lower immunization dose (10 μg) (Fig. 4g); however, at the higher immunization dose (50 μg) both CD4 and CD8 T-cell epitopes were recognized by T cells (Fig. 4h); there was also proliferation in response to OVA. Significant differences ($P < 0.05$) were detected in CD4 T-cell responses between the 10 and 50 μg DNA doses of OxMan-PLL on days 2 and 3 of the proliferation assay.

ELISPOT assay

The *ex vivo* 18-hr ELISPOT assay does not require cell expansion as it detects specifically activated effector cells (both CD4 and CD8 cytokine-producing terminal effectors). The sensitivity of the assay is higher than that of limiting dilution analysis, fluorescence-activated cell sorter (FACScan) analysis or ELISA methods and it can reliably detect precursor frequencies of antigen-specific effectors of 1 in 500 000 cells.[21–26] It is therefore an appropriate method for detection of antigen-specific cells at low precursor frequencies, as demonstrated previously in malaria models,[21–26] and thus cytotoxic T-cell assays were not performed. IFN-γ secretion by T cells in mice after two injections was measured by ELISPOT assay. The IFN-γ cytokine secretion reflected the results of the proliferation assay, in that, at the lower immunization dose (10 μg), OxMan-PLL-OVA induced primarily CD8 T-cell responses and RedMan-PLL-OVA CD4 T-cell responses (Fig. 5), but at the higher dose (50 μg) both $CD4^+$ and $CD8^+$ T cells secreted IFN-γ from either OxMan-PLL-OVA or RedMan-PLL-OVA immunized mice (Fig. 5). At the lower injection dose (10 μg), low levels of CD8 T cells were induced after RedMan-PLL-DNA immunization. The doses of 10 and 50 μg represent the amounts of DNA in the RedMan-PLL-DNA and OxMan-PLL-DNA complexes, respectively. Significant differences ($P < 0.005$) in the CD8 T-cell response of RedMan-PLL at the 10 and 50 μg DNA doses were observed. PLL-DNA and DNA alone did not induce T cells that recognized either the CD4 or the CD8 epitope peptides. In the ELISPOT assays,

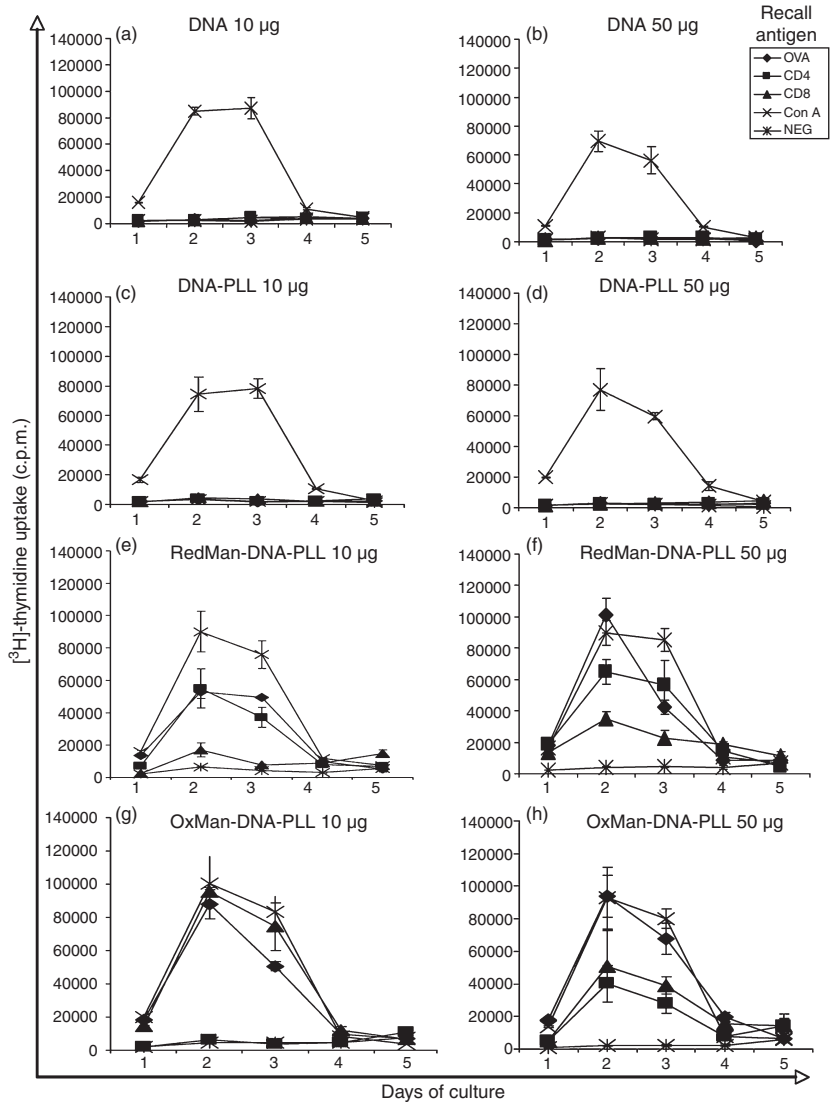

Figure 4. Proliferative responses in C57BL/6 mice immunized with different DNA vaccine formulations. C57BL/6 mice were immunized intradermally (i.d.) on days 0 and 14 with 10 μg ($R = 0.4$) or 50 μg ($R = 2.0$) oxidized mannan–poly L-lysine–ovalbumin DNA (OxMan-PLL-OVA DNA) or reduced mannan–poly L-lysine–ovalbumin DNA (RedMan-PLL-OVA DNA). Subsequently, 10–14 days after boost immunization, splenocytes were assessed for proliferation response to OVA (diamonds), OVA CD4 (squares) or CD8 T-cell epitopes (triangles) on days 1–5. Medium alone was used as a negative control ('NEG'; crosses). (a) DNA 10 μg; (b) DNA 50 μg; (c) DNA-PLL 10 μg; (d) DNA-PLL 50 μg; (e) RedMan-PLL-DNA 10 μg; (f) RedMan-PLL-DNA 50 μg; (g) OxMan-PLL-DNA 10 μg; (h) OxMan-PLL-DNA 50 μg. Concanavalin A (Con A) was used as an internal positive control. Data are shown as mean counts per minute (c.p.m.) for triplicate wells ± standard error of the mean. One representative of two experiments is shown ($n = 3$–4 mice/group).

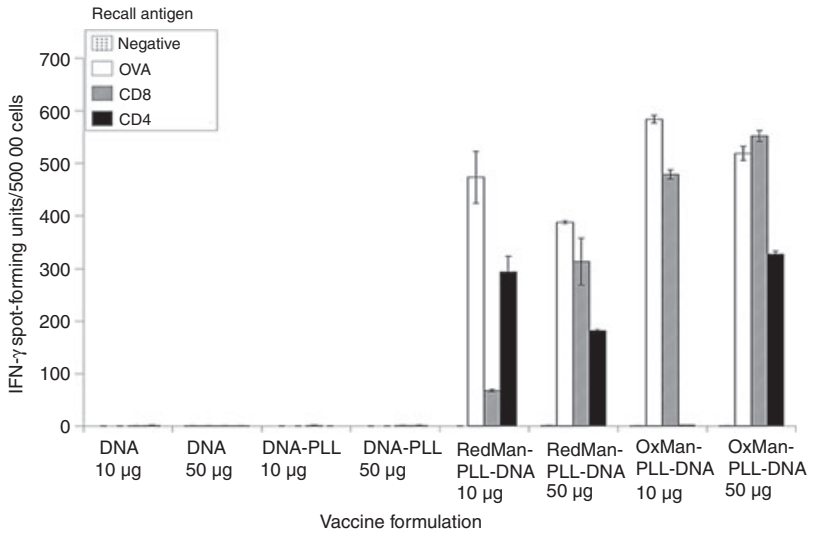

Figure 5. Interferon (IFN)-γ responses in C57BL/6 mice immunized with different DNA vaccine formulations. Mice were immunized intradermally (i.d.) on days 0 and 14 with 10 μg ($R = 0.4$) or 50 μg ($R = 2.0$) oxidized mannan–poly L-lysine–ovalbumin DNA (OxMan-PLL-OVA DNA) or reduced mannan–poly L-lysine–ovalbumin DNA (RedMan-PLL-OVA DNA). Subsequently, 10–14 days after boost immunization, IFN-γ responses to OVA (OVA), SIINFEKL (CD8) and OVA323-339 (CD4) were assessed by enzyme-linked immunosorbent spot-forming cell assay (ELISPOT). Mean spot-forming units for triplicate samples ± standard error are shown. This is a representative example of four different experiments ($n = 3$ mice/group).

precursor frequencies of IFN-γ-producing CD4 and CD8 splenocytes of 300 s.f.u./500 000 cells = 1/1666 and 500 s.f.u./500 000 cells = 1/1000 were observed, and precursor frequencies of > 1/10 000 have been demonstrated in a number of studies to correlate with protection.[13–17,22] Similarly high frequencies of stimulated cells were also found in the proliferation assays, where the peak response was at days 2–3 of culture.

In both proliferation and ELISPOT assays, non-conjugated OxMan and RedMan were mixed with DNA (10 and 50 μg) (without PLL linker) and injected into mice. Neither induced T cells, and the levels of IFN-γ were similar to those of other controls (data not shown). This demonstrates that OxMan or RedMan needs to be complexed to DNA via PLL and on its own does not induce responses. It is clear that both OxMan and RedMan-PLL-DNA can generate cellular (CD4 and CD8 T cell) immune responses depending on the dose injected.

ELISA

ELISA was used to detect the level of antibodies (total IgG) present in each mouse. Non-immunized mice (naïve) were used as controls (Fig. 6). Mice immunized with DNA-PLL, RedMan-PLL-DNA or OxMan-PLL-DNA did not produce antibodies at the 10 μg dose. However, at 50 μg, mice immunized with DNA-PLL, RedMan-PLL-DNA or OxMan-PLL-DNA produced weak antibodies (Fig. 6). It is clear that OxMan and RedMan-PLL-DNA induce weak humoral responses and differential cellular immune responses.

Tumour challenge

Ten days after the second injection, mice were challenged with a subcutaneous dose of 1×10^7 EG7 (EL4-OVA) tumour cells. All tumours grew in naïve mice and in mice immunized with 10 or 50 μg PLL-DNA (Fig. 7). In mice immunized with 10 or 50 μg RedMan-PLL-DNA, one of six tumours grew (Fig. 7). In mice immunized with 10 μg OxMan-PLL-DNA, two of six tumours grew, and in mice immunized with 50 μg OxMan-PLL-DNA none of six tumours grew. The mean tumour sizes in mice treated with RedMan-PLL-DNA (10 μg), RedMan-PLL-DNA (50 μg) and OxMan-PLL-DNA (50 μg) were all significantly smaller than in the PBS and PLL-DNA treated mice ($P < 0.001$). To demonstrate that the tumour growth inhibition was antigen specific, mice immunized with OxMan-PLL-DNA (50 μg) or control mice (PBS) were challenged with wild-type EL4 tumour cells and mice were not protected (Fig. 7). Our data indicate that

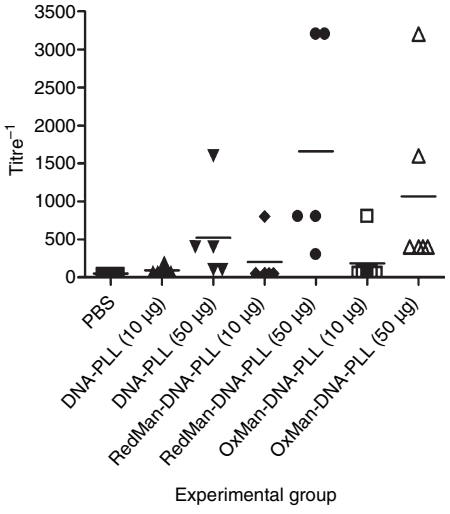

Figure 6. Antibody responses in C57BL/6 mice immunized with different DNA vaccine formulations. C57BL/6 mice were immunized intradermally (i.d.) on days 0 and 14 with 10 μg ($R = 0.4$) or 50 μg ($R = 2.0$) oxidized mannan–poly L-lysine–ovalbumin DNA (OxMan-PLL-OVA DNA) or reduced mannan–poly L-lysine–ovalbumin DNA (RedMan-PLL-OVA DNA) and, 14 days after boost injection, serum was collected and immunoglobulin G (IgG) levels were assessed by enzyme-linked immunosorbent assay (ELISA). Individual mouse titres are shown with means denoted by a horizontal line. A representative example of three different experiments ($n = 5$–6 mice/group) is shown.

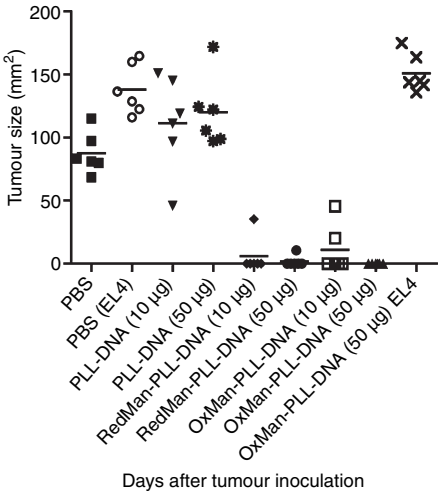

Figure 7. EG7 tumour challenge of C57BL/6 mice. C57BL/6 mice were immunized with 10 μg poly L-lysine (PLL)-DNA, 50 μg PLL-DNA, 10 μg reduced mannan (RedMan)-PLL-DNA, 50 μg RedMan-PLL-DNA, 10 μg oxidized mannan (OxMan)-PLL-DNA or 50 μg OxMan-PLL-DNA, and challenged with 1×10^7 EG7 cells. The tumour sizes for individual mice on day 9 after challenge are shown. In addition, two groups of mice immunized with phosphate-buffered saline (PBS) or 50 μg OxMan-PLL-DNA were challenged with EL4 cells as controls to demonstrate ovalbumin (OVA)-specific anti-tumour responses in immunized mice. The tumour sizes for individual mice on day 9 after challenge are shown. The horizontal bar denotes mean tumour size (in mm^2). Representative example of three different experiments ($n = 6$–7 mice/group) is shown.

OxMan-PLL-DNA and RedMan-PLL-DNA complexes generated OVA-specific protective immune responses in C57BL/6 mice.

Discussion

DNA vaccines have the potential to be an inexpensive and safe method of inducing protection against a range of diseases and of delivering genes into cells. Although good immune responses to DNA vaccines have been induced both in animal models and in human clinical trials, immunogenicity in humans still requires optimization.[27] Receptor-mediated uptake of DNA allows site-specific gene delivery to target cells. Several ligands binding to cell surface receptors have been used to transfect DNA into cells. Receptors include transferrin,[28] neurotensin,[8] low-density lipoprotein,[29] mannose[30,31] and asialoglycoprotein receptor.[32]

The mannose receptor is expressed on DCs and macrophages.[33] We previously demonstrated that the RedMan-MUC1 fusion protein induced predominantly Th2 responses whereas OxMan-MUC1 induced Th1 responses.[15] The effect of OxMan, as a result of the aldehydes present, enabled fast tracking of proteins through the endosomes to the cytosol.[18] Thus, OxMan and RedMan were used as carriers for DNA (using PLL as a linker) in this study.

As the interaction between OxMan-PLL or RedMan-PLL and DNA is ionic, the major factors that would affect the formation of complexes appropriate for efficient gene transfer include the molar charge ratio of the negatively charged phosphate groups of DNA molecules and the positively charged amine groups of the polycation, and the NaCl concentration at which complexes are formed. For the interaction of PLL with DNA, these factors affect the size and conformation of the complex (aggregated, condensed and relaxed),[34] which ultimately determine its ability to be endocytosed. In this study, the uptake of the complex was receptor-mediated via the mannose receptor, although other receptors that bind mannan cannot be excluded. Complexes were subsequently chosen to form at 0.7 M NaCl, as suggested in a similar study using mannose-PLL to target DNA to macrophages.[5] Analysis of OxMan-PLL-DNA conjugates at different R values and at 0.7 M NaCl indicated that the R value of the complex affects the proportion of DNA complexed (Fig. 2). We chose $R = 0.4$ and $R = 2.0$ for further study, in the light of the differences in migratory ability for these values in agarose gels resulting from different degrees of complexation, although both R values demonstrated complexation between DNA and PLL. Conjugation of PLL to OxMan was found to be necessary for complex formation because there were no signs of DNA retardation on the gel when a mixture of DNA and OxMan was analysed by gel electrophoresis. Polycations are toxic to cells, and it was interesting that conjugation to OxMan or RedMan reduced cytotoxicity, probably as a result of the neutralization of the positive charges of PLL by the reaction with OxMan or RedMan (Fig. 3). In previous studies using mannose-PLL-DNA or mannose-PEI-DNA, *in vitro* transfection efficiencies of 1–10% were obtained.[5,35] In our studies, OxMan-PLL-DNA and RedMan-PLL-DNA transfection efficiencies of 10% were obtained when the mannose-receptor-positive J774 macrophage cell line and bone-marrow-derived DCs were used (data not shown). We decided to initiate *in vivo* studies without carrying out extensive *in vitro* transfection studies, as in several studies no correlation was found between the *in vitro* and *in vivo* transfection efficiencies.[2,36–38]

Immunization of mice with 10 μg OxMan-PLL-DNA and RedMan-PLL-DNA ($R = 0.4$) induced primarily CD8 and CD4 responses, respectively (proliferation and ELISPOT assays); at the 50 μg DNA dose, OxMan-PLL-DNA and RedMan-PLL-DNA ($R = 2.0$) induced both CD8 and CD4 responses. The differential immune responses noted with OxMan-PLL-DNA and RedMan-PLL-DNA are common for proteins but not for DNA. For DNA vaccines, such deviations are only possible if there is coexpression of cytokines or costimulatory molecules. Previous studies demonstrated that DNA complexed to mannose-PLL (PLL chemically modified with single mannose units) was able to transfect macrophages with luciferase and galactosidase *in vitro* and *in vivo* for gene therapy and therefore no immune responses were measured.[5] In addition, transfection of human blood monocyte-derived macrophages with mannose-PLL-DNA (luciferase reporter gene) gave a high transfection efficiency and drove luciferase gene expression.[39] In another study, the gene encoding human α1-antitrypsin was delivered to macrophages by targeting the mannose receptor, and the α1-antitrypsin transcript was detected 48 hr after transfection.[40] Furthermore, mannose-PLL and mannose-PEI-DNA complexes have been used to deliver green fluorescent protein DNA into DCs.[35,41] In our studies, DNA alone (naked DNA) and DNA-PLL (Figs 4 and 5) did not induce T-cell responses (ELISPOT and proliferation assays). It was previously reported that sOVA-C1 DNA elicited immune responses *in vivo* at a 100 μg immunization dose after intramuscular injection.[19] In our studies, sOVA-C1 DNA was injected intradermally and, at either the 10 or 50 μg dose, DNA alone was ineffective at inducing a detectable cellular immune response, although weak antibodies were detected (Fig. 6). At these doses, when DNA was complexed to either RedMan or OxMan, strong immune responses were induced. Hence, intradermal injection, targeting antigen-presenting cells, in the presence of RedMan or OxMan enhances immune responses to DNA. Thus, targeting DNA to C-type lectins (i.e. the mannose receptor) using mannan (in the oxidized or reduced form) is superior to DNA alone or DNA-PLL, at least at

these immunization doses (10 and 50 μg) and with this route of immunization (intradermal).

It is clear that both OxMan and RedMan could induce differential immune responses depending on the dose injected; however, all groups showed similar tumour protection. We previously demonstrated that aldehydes present in OxMan allowed rapid escape from endosomes into the cytoplasm and induced high levels of tumour necrosis factor (TNF)-α and interleukin (IL)-12 production by antigen-presenting cells compared with RedMan.[42] It was originally thought that OxMan would disrupt the endosomal compartment more efficiently than RedMan, allowing release of the DNA into the cytoplasm for subsequent nuclear transport, and thus would protect the DNA from degradation in the lysosomes. This assumption was based on our previous findings that aldehydes present in OxMan allow fast tracking from the endosomes to the cytoplasm. However, as OxMan-PLL-DNA and RedMan-PLL-DNA both induced differential immune responses and protection in the *in vivo* model, endosomal escape alone cannot explain these results. However, despite the absence of aldehyde groups in RedMan, secondary amines generated by borohydride reduction could make the complex endosomolytic. We are currently investigating *in vitro* transfection, *in vivo* trafficking and the mechanism of action. Recently, we demonstrated that both OxMan and RedMan are able to activate DCs to a mature phenotype and induce high levels of IL-12 and IFN-γ production by DCs, and thus have further effects on the nature of the immune response generated.[43] Thus, it is likely that a number of mechanisms are involved in the differential induction of immune responses by OxMan-PLL-DNA and RedMan-PLL-DNA. Furthermore, RedMan or OxMan-PLL-DNA complexes were injected intradermally in our studies in order to target C-type lectins (i.e. the mannose receptor) on DCs. In most studies to date in which DNA alone was used, DNA was injected intramuscularly and thus different cells were targeted. It is of relevance that, in our recent studies using a MUCI plasmid, MUC1, DNA alone induced weak cellular and antibody responses which were enhanced when the DNA was complexed to either OxMan or RedMan. Thus, the use of mannan as a carrier for DNA to target C-type lectins (such as the mannose receptor) enhances immune responses *in vivo* in another model system (C. K. Tang, G. Pietersz & V. Apostolopoulos, manuscript in preparation). PEI has been used as an alternative polycationic carrier to PLL, with endosomolytic properties, for *in vitro* and *in vivo* gene delivery. Use of DNA complexes of OxMan and RedMan conjugates of PEI should further increase the efficiency of DNA delivery.[35,41]

Our data suggest that OxMan-PLL and RedMan-PLL are good candidates for efficient non-viral, receptor-mediated gene transfer ligands and are far superior to DNA alone or DNA-PLL. Unlike other methods of gene delivery (e.g. microparticles or gene gun), OxMan and RedMan are cheap to produce, there is no need for special equipment for inoculation and safety has been demonstrated in more than 300 patients.[44–48] Hence, OxMan-PLL and RedMan-PLL represent an attractive novel form of receptor-mediated gene transfer for cancer immunotherapy studies.

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