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# Oxidized and reduced mannan mediated MUC1 DNA immunization induce effective anti-tumor responses

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ABSTRACT

DNA immunization is an attractive form of vaccination, which has shown promising results only in small animal models. There is a need to develop efficient gene delivery systems. We previously demonstrated that oxidized (OM) and reduced mannan (RM) complexed to ovalbumin DNA via poly-L-lysine (PLL), were able to generate potent immune responses in mice. Herein, we further investigated the suitability of OMPLL and RMPLL as carriers for mucin 1 (MUC1) DNA vaccination for cancer immunotherapy. Studies presented here showed that immune responses in C57BL/6 mice induced by OMPLL-MUC1 DNA and RMPLL-MUC1 DNA immunization were more immunogenic compared to MUC1 DNA alone. Moreover, tumor protection was evident at a dose as low as 0.5 μg. In addition, strong T cell responses were induced in HLA-A2 transgenic and human MUC1 transgenic mice. These results demonstrate the potential of OM and RM as efficient non-viral gene delivery carriers for DNA vaccines for use in cancer immunotherapy.


## 1. Introduction

DNA vaccines have many potential advantages over earlier generation vaccines such as attenuated and subunit vaccines. Numerous studies have aimed to improve the immunogenicity of DNA vaccines, which include the incorporation of ligands that targets receptors on cells, termed receptor mediated gene delivery [1]. This form of delivery is based on ionic interaction between the receptor specific ligand and DNA to form a complex. To enable this interaction, cationic polymers are conjugated to the ligand so that it can interact ionically with the negatively charged DNA [2]. This serves a dual function of bridging the two components and the condensation of DNA into compact and uniform structures without the need for further chemical modifications. For instance, a complex formed between asialoorosomucoid coupled to poly-L-lysine (PLL) and chloramphenicol acetyltransferase expressing DNA plasmid was able to transfect HepG2 cells [3]. PLL has also been used as a linker in mannose-based complexes [4–7] and other ligands such as glycosaminoglycans [8] and neurotensin [9].

Recently, we demonstrated that mannan was able to increase the immunogenicity of ovalbumin (OVA) DNA [10]. OVA expressing DNA plasmids complexed to oxidized (OM) or reduced mannan (RM), via PLL as a linker (OMPLL or RMPLL), could generate specific immune responses *in vivo* and protect mice from B16-OVA melanoma challenge [10]. In addition, depending on whether mannan was in an oxidized or reduced state, the *in vivo* immune response was steered towards a Th1 or Th2 response. This immune directing property of OM and RM was previously noted in early pre-clinical studies using MUC1 fusion protein (MUC1FP) [11–15]. In phase I/II and pilot phase III clinical trials with OM-MUC1FP, humoral and/or cellular responses are induced in adenocarcinoma patients [16–19].

MUC1 is a tumor associated antigen that is expressed on adenocarcinomas. It is natively present on the apical surface of epithelial cells lining the secretory ducts. Immunotherapy studies that targets MUC1-expressing tumor cells have involved immunization with peptides predominantly from the protein core in combination with carriers or adjuvants. In more recent years, MUC1 immunization studies have extended to immunizing with DNA [20], RNA [21], viruses [22] and carcinoma/dendritic cells (DC) fusions [23]. MUC1 DNA immunization has been actively investigated since the demonstration of tumor protective immune response after intramuscular injection of naked MUC1 DNA [24]. Attempts to augment these responses has led to the use of adjuvants such as Flt-3 ligand

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[25], GM-CSF [20] and IL-18 [26], co-injected or co-expressed in the plasmid.

In this study, we investigate the immunogenicity of human MUC1 DNA complexed to OMPLL or RMPLL by immunizing C57BL/6, HLA-A* 201/$K^b$ (HLA-A2) transgenic and human MUC1 transgenic mice. Herein, we demonstrate that strong cellular and humoral responses were induced in C57BL/6, HLA-A2 transgenic and MUC1 transgenic mice immunized with OMPLL-MUC1 DNA and RMPLL-MUC1 DNA. Prophylactic and therapeutic protective responses against $MUC1^+$ tumors in C57BL/6 mice were also induced. These studies validate the use of OM and RM in MUC1 DNA-based vaccines for cancer immunotherapy.

## 2. Materials and methods

### 2.1. Materials

Complete RPMI 1640 media was prepared by supplementing plain RPMI 1640 with 2% HEPES, 0.1 mM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 10% fetal calf serum. For *in vitro* re-stimulation the following antigens were used: MUC1FP (MUC1 fusion protein; a Glutathione-*S*-transferase tagged fusion protein comprising of 5 variable number of tandem repeats (VNTR) and STAPPAHGV peptide, a HLA-A2 CD8 T cell epitope [13]. Endotoxin-free MUC1FP was prepared as described previously [11,16]. The HLA-A2 restricted $MUC1\ CD8^+$ epitope, STAPPAHGV peptide [13], was synthesized by GensScript (Piscataway, NJ, USA) and its purity was >95% as determined by pure by mass spectrometry and high performance liquid chromatography (HPLC).

### 2.2. DNA plasmids

MUC1 DNA plasmid used in this study was prepared by subcloning the full length human MUC1 comprising of 22 tandem repeats with the C- and N-terminus into the expression vector pcDNA3.1 [27]. MUC1 plasmids were purified using the Qiagen Endofree Plasmid Maxi Kit (Qiagen, Hamburg, Germany) according to the manufacturer's instructions.

### 2.3. Mice and immunization

C57BL/6 mice were purchased from Walter Eliza Hall Institute. HLA-A*0201/$K^b$ transgenic mice (HLA-A2 and H-$2^b$) [28] and human MUC1 transgenic mice (H-$2^d$) [29] were bred and maintained in the animal house facilities of Burnet Institute (Heidelberg, Victoria, Australia). Mice (8–12-week old) were immunized twice on days 0 and 14 intradermally (ID) at the base of tail.

### 2.4. Conjugation of OM and RM to PLL

To prepare OM, 14 mg mannan derived from *Saccharomyces cerevisiae* (SIGMA, St. Louis, USA) was dissolved in 1 ml sodium phosphate buffer (pH 6, 0.1 M), followed by the addition of 100 μl 0.1 M sodium periodate (dissolved in pH 6 phosphate buffer) and incubated at 4 °C for 1 h in the dark. Ten microliters ethanediol was added to the mixture which was then incubated for 30 min at 4 °C. The mixture was passed through a PD-10 column (pre-equilibrated in 5 mM NaCl solution) and OM collected in the first 2 ml after the void volume (2.5 ml). One milligram PLL which has an $M_r$ of 27,400 was (SIGMA, Steinheim, Germany) dissolved in phosphate buffered saline (PBS), was added to 2 ml OM. OMPLL was generated in the dark at room temperature (RT) over night (O/N). RMPLL was prepared by adding 1 mg sodium borohydride to OMPLL for 3 h at RT before dialysis into 5 mM NaCl. The final concentrations of mannan and PLL in both OMPLL and RMPLL were 7 mg/ml and 0.5 mg/ml, respectively.

### 2.5. RMPLL and OMPLL complexation to MUC1 DNA

OMPLL and RMPLL were complexed to MUC1 DNA as previously described [10]. Briefly, the amount of the carrier added to MUC1 DNA was determined by the specified *R* value, which was defined as the molar charge ratio of DNA ($PO_4^-$) to PLL ($NH_3^+$). Carriers (OMPLL, RMPLL and PLL) in double distilled water were added to an equal volume of MUC1 DNA. This was followed by incubation at 37 °C for 2 h before the complexes were used. The degrees of complexation between OMPLL and MUC1 DNA of varying *R* values ($R = 10, 5, 2, 1, 0.4$ and 0.1) were compared via the extent of DNA retardation in 0.6% (w/v) agarose gel electrophoresis for 1 h at 100 mV.

### 2.6. ELISpot assay

Ten to 14 days after the last immunization, spleens were removed and immune responses assessed. Splenocytes from immunized mice were collected and treated with 0.73% $NH_4Cl$ for 10 min at 37 °C to lyse red blood cells. IFN-γ ELISpot assays were performed on MultiScreen-IP Filter Plates (MAIP S45 10) with hydrophobic PVDF filters (Millipore, UK) whilst IL-4 ELISpots were performed on MultiScreen-HA Filter Plates (MAHA S45 10) with mixed cellulose esters filters (Millipore, UK). MAIP S45 10 plates were pre-wet with 50 μl of 70% (v/v) ethanol, washed 6 times with 200 μl sterile PBS and coated with 70 μl of 5 μg/ml anti-IFNγ capture antibody, AN18 (Mabtech, Australia) in PBS and incubated at 4 °C O/N. For IL-4 ELISpot assays, 70 μl of 5 μg/ml anti-IL-4 capture antibody (Mabtech, Australia) was added to MAHA S45 10 plates and incubated at 4 °C for 18 h. Both IFN-γ and IL-4 detection plates were washed 6 times with sterile PBS and blocked with 200 μl of RPMI media for 2 h at 37 °C. $5 \times 10^5$ splenocytes in 100 μl complete media were incubated with 20 μg/ml MUC1FP and STAPPAHGV peptide or media alone for 18 h in IFN-γ ELISpots and 48 h in IL-4 ELISpot. ConA (1 μg/ml) was used as an internal positive control. Plates were submerged in double distilled $H_2O$ to lyse remaining cells and developed by incubation with biotinylated anti-IFNγ antibody, R46A2 (Mabtech, Australia) or biotinylated anti-IL-4 antibody (Mabtech, Australia) for 2 h at RT followed by 1 μg/ml streptavidin-alkaline phosphatase (Sigma, Australia) for 30 min at RT. Spots were visualized by using a colorimetric Alkaline Phosphatase kit (BioRad, USA) and counted using an AID ELISpot reader (Autoimmun Diagnastika GmbH, Germany). Data are presented as mean spot forming units (SFU) per $5 \times 10^5$ cells ± standard error of mean (S.E.M.).

### 2.7. ELISA

Mice were bled 14 days after the final immunization and sera collected after centrifugation at 13,000 rpm at 4 °C for 20 min. Polyvinyl chloride micro-titre plates (Corning, Massachusetts, USA) were coated with 10 μg/ml MUC1FP in 0.05 M sodium bicarbonate, pH 9.6, and incubated at 4 °C for 18 h and washed with 0.05% Tween-20/PBS. Plates were blocked with 2% (w/v) bovine serum albumin (BSA)/PBS for 2 h at 37 °C and washed with 0.05% (v/v) Tween-20/PBS, followed by incubation with serially diluted mouse sera in PBS for 2 h at RT. After washing with 0.05% (v/v) Tween-20/PBS, 1 μg/ml of horseradish–peroxidase-conjugated sheep anti-mouse Ig (Sigma, Australia) in PBS was added per well and incubated for a further 2 h at RT. After wash, plates were developed by addition of 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) solution, which was a mixture of 5 ml ABTS buffer (1 M sodium hydrogen phosphate, 1 M citric acid, pH 4.5), 100 μl ABTS stock (0.5 mg/ml) and 4 μl

$H_2O_2$ per plate. Plates were read after 15–30 min using Fluostar Optima microplate reader (BMG labtech, Offenburg, Germany) at absorbance of 405 nm. Data are presented as mean ± S.E.M. ($n = 3$).

### 2.8. Prophylactic and therapeutic tumor studies

In prophylactic tumor protection studies, groups of mice (6 per group) were immunized twice (at a 2-week interval) before being challenged with $1 \times 10^6$ B16-MUC1 cells resuspended in 100 μl sterile PBS (a gift from Dr. Jianlin Gong, University of Boston, USA) by subcutaneous injection into the abdomen. High levels of MUC1 expression was maintained by culturing B16-MUC1 cells in 1.2 mg/ml G418/gentamycin (Invitrogen, California, USA). For therapeutic studies, mice were injected subcutaneously with $1 \times 10^5$ B16-MUC1 tumor cells and subsequently immunized ID at the base of tail with various DNA complexes (RMPLL-, OMPLL-MUC1 DNA or MUC1 DNA alone) on days 3 and 7 after inoculation of B16-MUC1 cells. Tumor growth was monitored by measuring tumor area (length × width).

### 2.9. Detection of MUC1 expression on B16-MUC1 tumor cells

Tumors were removed from mice at the end of tumor challenge experiments. Single suspensions of tumor cells were prepared by teasing and flushing the tumor mass with RPMI media followed by treatment with 0.73% $NH_4Cl$ for 10 min at 37 °C to lyse red blood cells. Cells were washed, resuspended in RPMI media and cultured for 7 days. MUC1 expression on adherent cells was determined by staining with anti-MUC1 monoclonal antibody (BC2) [30] for 45 min at 4° followed by the addition of anti-mouse F-$(ab')_2$-FITC antibody (Chemicon, Melbourne, Australia) for a further incubation of 45 min at 4 °C. MUC1 expression was detected using flow cytometry (FACS Canto, NJ, USA).

### 2.10. Statistics

All data are represented as means ± standard error of mean. One-way analysis of variance (ANOVA) with Bonferroni's post-test was used to compare significant difference between 3 or more groups. Log-rank (Mantel-Cox) Test was used to analyse the significant difference between survival curves. $p$-Value threshold of $p \leq 0.05$ indicates a statistically significant difference. $p \leq 0.01$ indicates a high significant difference.

## 3. Results

### 3.1. RMPLL-MUC1 DNA and OMPLL-MUC1 DNA induce specific T cell and antibody responses in C57BL/6 mice

The ability of RMPLL and OMPLL to form complexes with DNA plasmids was assessed by gel retardation assay (Fig. 1). It was observed that the complexation process was dependent on the ratio of the $PO_4^-$ groups in DNA to the $NH_3^+$ groups in the polyamines ($R$ value). The degree of complexation was manipulated by adjusting the $R$ value, as shown in Fig. 1. OMPLL-MUC1 DNA complexes were formed over a range of $R$ values (0.1–10). At $R = 0.1$ there is 10 times more $NH_3^+$ than $PO_4^-$, while there is 10 times more $PO_4^-$ than $NH_3^+$ at $R = 10$. It was observed that the degree of retardation decreased as the $R$ value increased indicating weaker DNA binding at higher $PO_4^-$:$NH_3^+$ ratios.

To determine whether OMPLL-MUC1 DNA or RMPLL-MUC1 DNA was able to induce T cell responses in MUC1 in C57BL/6 mice, groups of 3 mice were immunized with 10 μg DNA doses ($R = 0.4$). Such a dosage based on our previous experience with OMPLL-OVA DNA and RMPLL-OVA DNA immunization studies [10]. IFN-γ and IL-4 secretion by T cells in mice after 2 injections was measured by ELISpot assay. The ELISpot assay is an highly sensitive assay that can reliably detect precursor frequencies of antigen specific effectors of 1 in every 500,000 cells. It is therefore an appropriate method to detect antigen specific cells at low precursor frequencies in malaria [31], human papillomavirus [32], melanoma [33] and MUC1 models [34].

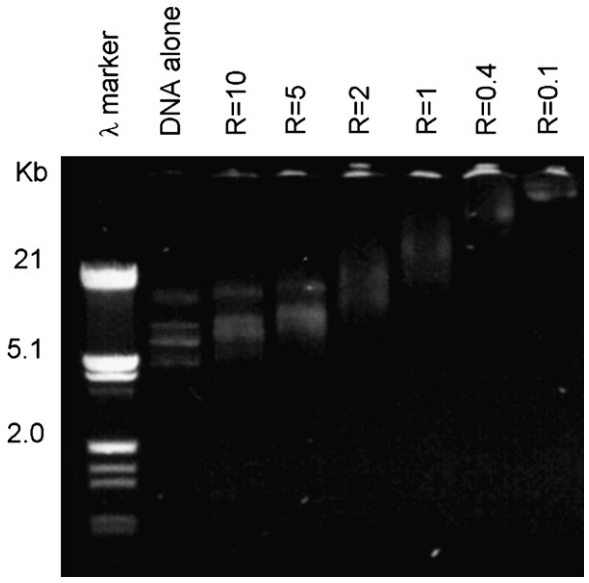


**Fig. 1.** Complexation efficiency between carrier (OMPLL) and MUC1 DNA. Degree of complexation between OMPLL and MUC1 DNA was assessed by the detection of loss of negative charges in DNA compared to uncomplexed DNA (DNA alone) using 0.6% agarose gel electrophoresis. OMPLL-OVA DNA complexes formed at successive $R$ values ($R = 0.1$–10) are shown.

IFN-γ producing specific T cell responses to MUC1 was generated in all immunized groups using MUC1FP as a recall antigen. However, the level of response induced was different between groups. Immunization with MUC1 DNA alone induced moderate IFN-γ responses (142 SFU) compared to the PBS group ($p \leq 0.05$) (Fig. 2A). This response was greatly enhanced in OMPLL-MUC1 DNA and RMPLL-MUC1 DNA immunized groups. The IFN-γ responses induced by OMPLL-MUC1 DNA (521 SFU) and RMPLL-MUC1 DNA (379 SFU) were significantly higher than MUC1 DNA alone ($p \leq 0.01$). Interestingly, the pattern of the IL-4 response was different compared to that of the IFN-γ response (Fig. 2B). MUC1 DNA alone and OMPLL-MUC1 DNA immunized groups did not induce IL-4 responses, while RMPLL-MUC1 DNA immunized mice generated significant IL-4 responses ($p \leq 0.01$). Furthermore, IgG antibody responses against MUC1FP were only noted in RMPLL-MUC1 immunized mice (Fig. 2C). It is clear that both OMPLL and RMPLL complexation to MUC1 DNA augmented specific immune responses to MUC1, while the type of responses induced were different. OMPLL-MUC1 DNA-induced solely an IFN-γ response and RMPLL-MUC1 DNA generated both IFN-γ and IL-4 responses to MUC1.

### 3.2. OMPLL-MUC1 DNA and RMPLL-MUC1 DNA immunization protects mice in prophylactic tumor challenge and tumor therapy studies

To evaluate the efficacy of prophylactic tumor protection of the DNA complexes, C57BL/6 mice immunized with 10 μg MUC1 DNA alone, RMPLL-MUC1 DNA or OMPLL-MUC1 DNA were challenged subcutaneously with $1 \times 10^6$ MUC1 expressing B16 melanoma cells (B16-MUC1) on the abdomen (Fig. 3). All mice immunized

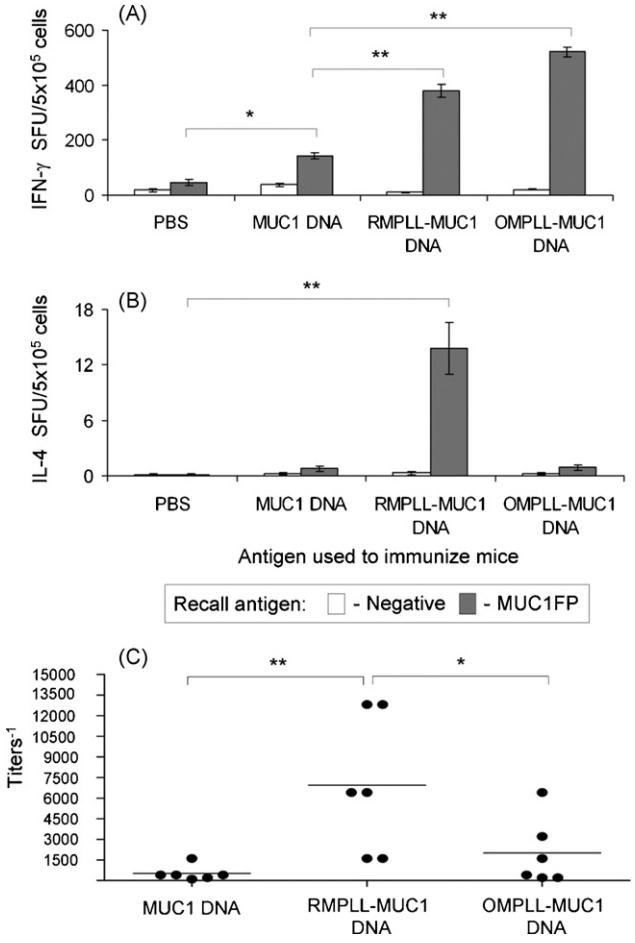


**Fig. 2.** (A) IFN-γ and (B) IL-4 cytokine production from splenocytes of C57BL/6 mice immunized with 10 μg MUC1 DNA alone, RMPLL-MUC1 DNA and OMPLL-MUC1 DNA in response to recall antigen, MUC1FP. Non-pulsed cells were used as a negative control and ConA (1 μg/ml), which induced >1000 SFU was used as an internal positive control (not shown). Mean spot forming units (SFU) of triplicate samples ± S.E.M. are reflected. A representative example of 2 different experiments ($n = 3$ mice/group) is shown. * and ** indicate significant differences of $p \leq 0.05$ and $p \leq 0.01$, respectively, compared to controls. (C) IgG antibody responses against MUC1FP in mice immunized with 10 μg MUC1 DNA dose of various complexes (MUC1 DNA alone, RMPLL-MUC1 DNA or OMPLL-MUC1 DNA). Data is presented as titres$^{-1}$ of individual mice. A representative example of two different experiments ($n = 3$ mice/group) is shown. * and ** indicates significant difference of $p \leq 0.05$ and $p \leq 0.01$, respectively.

with RMPLL-MUC1 DNA and OMPLL-MUC1 DNA showed retarded tumor growth compared to controls. Mice immunized with MUC1 DNA alone generated protective responses similar to OMPLL-MUC1 DNA and RMPLL-MUC1 DNA groups, and no significant difference in tumor size between OMPLL-MUC1 DNA (average, 28.5 mm$^2$), RMPLL-MUC1 DNA (average, 26.4 mm$^2$) and MUC1 DNA (average, 21.4 mm$^2$) on day 12 (Fig. 3A). As for the ELISpot data, while immunization with MUC1 DNA-induced significant T cell responses, the T cell response was greater in OM/RMPLL-MUC1 DNA groups. Therefore, to determine the dose at which differences in tumor protective responses could be observed between MUC1 DNA, RMPLL-MUC1 DNA and OMPLL-MUC1 DNA groups, mice were immunized with 5 μg (Fig. 3B) or 0.5 μg (Fig. 3C) MUC1 DNA dose, and challenged with $1 \times 10^6$ B16-MUC1 cells. A difference in protection between groups was evident at 5 μg DNA dose. At day 11, although mice immunized with MUC1 DNA (average, 48.1 mm$^2$) showed tumor protection, only OMPLL-MUC1 DNA (average, 13.4 mm$^2$; $p \leq 0.01$) and RMPLL-MUC1 DNA groups (average of 20.6 mm$^2$; $p \leq 0.01$) significantly suppressed tumor growth compared to PBS treated mice (average, 81 mm$^2$) (Fig. 3B). Even at 0.5 μg DNA dose, significant tumor protection was noted by immunization with OMPLL-MUC1 DNA (average, 37.7 mm$^2$; $p \leq 0.05$) and RMPLL-MUC1 DNA (average of 27 mm$^2$; $p \leq 0.05$) compared to PBS treated mice (average, 56.7 mm$^2$) (Fig. 3C); immunization with MUC1 DNA alone failed to induce any protection (average, 64.1 mm$^2$). This tumor protective response was shown to be specific by FACS analysis as there was lesser MUC1 expression present on the tumor cells of OMPLL-MUC1 DNA immunized mice compared to the PBS treated group (Fig. 3D). These results demonstrate the efficacy of OMPLL-MUC1 DNA and RMPLL-MUC1 DNA in inducing tumor protection than immunization with MUC1 DNA alone in C57BL/6 mice. In addition, a similar level of protection was observed in OMPLL-MUC1 DNA and RMPLL-MUC1 DNA immunized mice (Fig. 2).

To further study the tumor protective response generated by immunizing with the DNA complexes, a therapeutic study was performed. C57BL/6 mice were injected subcutaneously with $1 \times 10^5$ B16-MUC1 cells and, on days 3 and 7, mice were immunized with a 5 μg DNA dose of MUC1 DNA alone, RMPLL-MUC1 DNA or OMPLL-MUC1 DNA (Fig. 4), on the basis of the protection study as described in Fig. 3A. Mice with tumors over the size of 100 mm$^2$ were culled due to ethical limitations. Fig. 4 shows Kaplan Meier survival curves of mice from each group. Naïve mice were not protected and none survived past day 24. Tumor progression was slower in the DNA immunised groups. OMPLL-MUC1 DNA and RMPLL-MUC1 DNA immunised groups have a slightly higher level of protection compared to MUC1 DNA alone, with reference to the naïve mice. The pattern in the level of protection between groups appeared similar to that observed in prophylactic tumor challenge at the same DNA

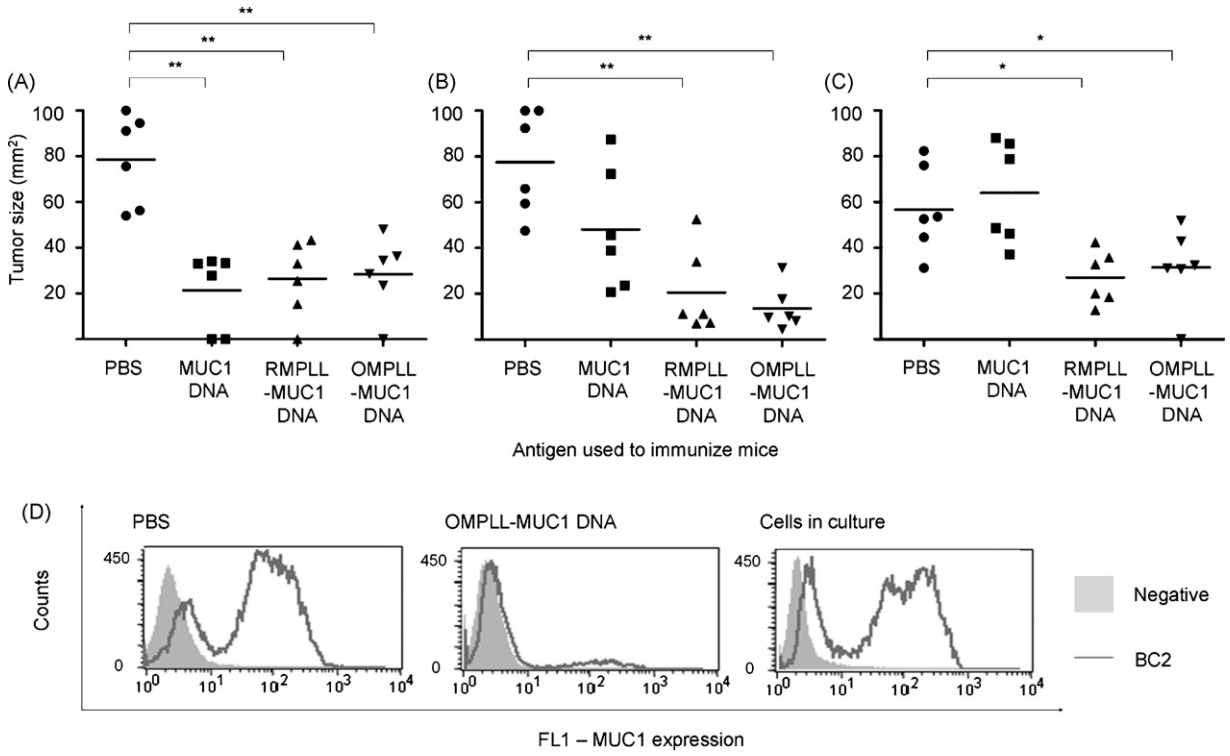


**Fig. 3.** Tumor size of mice immunized with (A) 10 μg, (B) 5 μg and (C) 0.5 μg DNA doses with either MUC1 DNA alone, RMPLL-MUC1 DNA or OMPLL-MUC1 DNA. Tumor sizes (as measured by the area of tumor) of individual mice on day 12 after B16-MUC1 challenge are shown. The horizontal bar denotes mean tumor size ($mm^2$). A representative example of two different experiments ($n = 6$–7 mice/group) is shown. * and ** indicate significant differences of $p \leq 0.05$ and $p \leq 0.01$, respectively, compared to the controls. (D) Expression of MUC1 on B16-MUC1 tumor cells from PBS injected mice, OMPLL-MUC1 DNA immunized mice and B16-MUC1 cells in culture. Single cell suspensions were prepared from tumor mass of mice in tumor challenge experiments at day 11 and cultured for 7 days before MUC1 expression was detected using flow cytometry. Data presented is representative of two separate experiments.

immunization dose. Collectively, results here indicate that compared to MUC1 DNA alone, OM/RMPLL-MUC1 DNA were able to generate enhanced tumor protective responses in prophylactic and therapeutic settings in C57BL/6 mice.

### 3.3. OMPLL-MUC1 DNA and RMPLL-MUC1 DNA induce strong T cell responses in HLA-A2 and MUC1 transgenic mice

HLA-A2 transgenic mice are commonly used in pre-clinical models for characterizing and assessing epitopes relevant to T cell

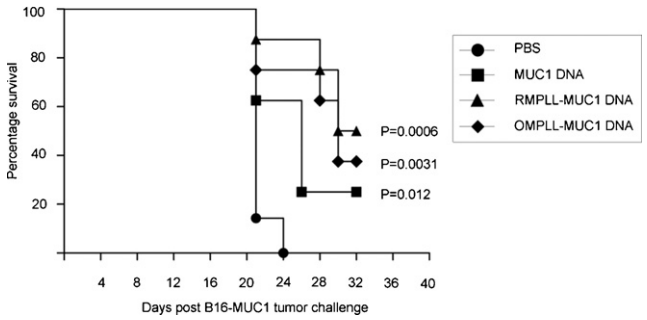


**Fig. 4.** Therapeutic efficacy of MUC1 DNA, RMPLL-MUC1 DNA and OMPLL-MUC1 DNA against B16-MUC1 in C57BL/6 mice. Mice were injected with $1 \times 10^5$ B16-MUC1 cells subcutaneously on the abdomen before being immunized with 5 μg MUC1 DNA, RMPLL-MUC1 DNA or OMPLL-MUC1 DNA on days 3 and 7. Tumor sizes (area $mm^2$) of individual mice were recorded every other day. Data is presented as survival curves of immunized groups (Kaplan Meier). *p*-Value of individual curve compared to the naïve group are reflected (log-rank test). Representative example of two different experiments ($n = 6$ mice/group) is presented.

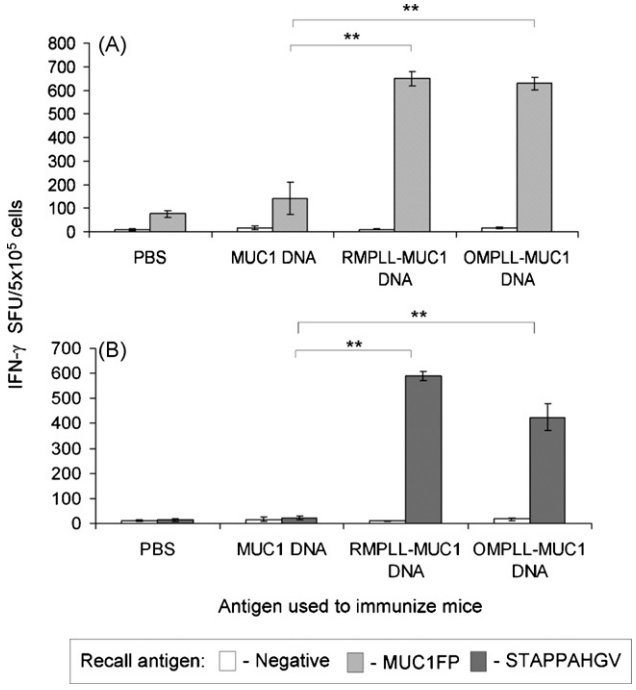


**Fig. 5.** IFN-γ ELISpot assay of splenocytes from immunized HLA-A2 transgenic mice. Secretion of IFN-γ by splenocytes in response to (A) MUC1FP and (B) STAPPAHGV (HLA-A2 CD8 T cell epitope). Non-pulsed cells were used as a negative control and ConA (1 μg/ml), which induced >1000 SFU was used as an internal positive control (not shown). Mean spot forming units (SFU) of triplicate samples ± standard error are shown ($n = 3$ mice/group). ** indicates significant difference of $p \leq 0.01$.

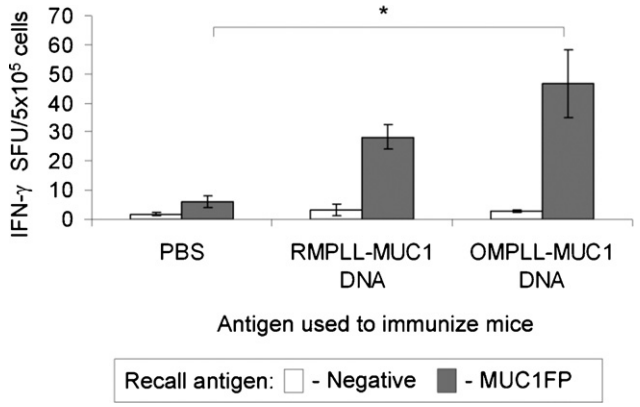


**Fig. 6.** IFN-γ ELISpot assay of splenocytes from immunized MUC1 transgenic mice. Secretion of IFN-γ by splenocytes in response to MUC1FP was determined by IFN-γ ELISpot. Splenocytes were incubated with MUC1FP at 20 μg/ml for 18 h before development of the assay. Non-pulsed cells were used as a negative control and ConA (1 μg/ml), which induced >1000 SFU was used as an internal positive control (not shown). Data is presented as mean spot forming units (SFU) of triplicate samples ± S.E.M. A representative example of two different experiments ($n = 3$ mice/group) is shown. * indicates a significant difference of $p \leq 0.05$.

recognition in humans. To determine whether RMPLL-MUC1 DNA or OMPLL-MUC1 DNA immunization (10 μg dose) could induce T cell responses in HLA-A2 transgenic mice, IFN-γ ELISpot assays were performed using MUC1FP or the human $CD8^+$ T cell epitope, STAPPAHGV [35], as recall antigens.

Using MUC1FP as a recall antigen, a similar response pattern was noted in immunized mice as compared to that evident in C57BL/6 mice (Fig. 2). OMPLL-MUC1 DNA ($p \leq 0.01$) and RMPLL-MUC1 DNA ($p \leq 0.01$)-induced stronger IFN-γ response than MUC1 DNA alone (Fig. 5A). In addition, OMPLL-MUC1 DNA or RMPLL-MUC1 DNA-induced strong IFN-γ responses against STAPPAHGV recall peptide (Fig. 5B); while MUC1 DNA immunized mice were not responsive to STAPPAHGV recall peptide (Fig. 5B). This observation indicates that CD8 T cells recognizing the HLA-A2 restricted T cell epitope were induced by immunization with OMPLL-MUC1 DNA and RMPLL-MUC1 DNA.

Next, to determine if OMPLL-MUC1 DNA and RMPLL-MUC1 DNA could break tolerance, human MUC1 transgenic mice were immunized with 10 μg DNA dose of OMPLL-MUC1 DNA or RMPLL-MUC1 DNA and T cell responses and assessed by IFN-γ ELISpot assays. IFN-γ secreting T cell responses to MUC1FP could be detected in RMPLL-MUC1 DNA and OMPLL-MUC1 DNA ($p \leq 0.05$) immunized MUC1 transgenic mice (Fig. 6). Results here indicate that OMPLL-MUC1 DNA and possibly RMPLL-MUC1 DNA immunization are able to break T cell tolerance in MUC1 transgenic mice and induce T cell responses.

## 4. Discussion

Cancer immunotherapy represents an attractive form of treatment against a ubiquitous and aggressive disease. Ideally, it would provide an all pervading targeted nontoxic and non-invasive mechanism to eradicate even micrometastatic disease. However, inducing potent immune responses against self-antigens expressed on cancer cells is a challenge. The ultimate goal in immunization strategies is to enable the presentation of antigenic peptides by APCs to T cells. The process leading up to this pivotal event requires the right conditions in order for the appropriate type of response to be mounted against tumor cells. DNA vaccination by endocytic receptor targeting is a strategy to enhance cellular uptake and antigen presentation. While immune induction is imperative, its efficacy in breaking tolerance, as seen in cancer patients, is most desirable.

Here, we demonstrate the ability of OMPLL and RMPLL to augment immunogenicity of DNA-based vaccines, using MUC1 encoding DNA. Parallel to observations in OVA DNA studies, immunizing C57BL/6 mice with OMPLL-MUC1 DNA and RMPLL-MUC1 DNA-induced superior immune responses compared to MUC1 DNA alone [10]. The augmented immune response rendered immunized mice more resistant to a B16-MUC1 tumor challenge. Such enhanced responses was more apparent when used in lower DNA doses, at which only OMPLL-MUC1 DNA and RMPLL-MUC1 DNA immunized groups showed significant reduction in tumor size compared to the naïve group. Therefore the efficacy of mannan mediated MUC1 immunization was in part dependent on the dose. A reduction in dosage from 10 μg to 5 μg reduced the level of T cell responses, resulting decreased level of tumor immunity. Interestingly, with a dose as small as 0.5 μg, mannan DNA complexes still induced a significant level of tumor growth inhibition while there was no protection in MUC1 DNA alone group. Overall, this demonstrates the efficacy of mannosylated DNA complexes in generating anti-tumor responses. Future experiments are focussed on improving the immunogenicity of OM and RM mediated DNA immunization by the addition of polycationic linkers with endosomolytic properties, such as polyethylenimine [36] and dendrimers [37]. Also noted in this study is the previously identified ability of OMPLL to induce a predominantly cell mediated response, and RMPLL to induce both humoral and cell mediated responses. OMPLL-MUC1 DNA-induced strong T cell responses against MUC1FP but a weak antibody response, while RMPLL-MUC1 DNA-induced both T cell responses and significant antibody responses against MUC1FP. Regardless of this difference, both carriers conferred a similar level of tumor protection to C57BL/6 mice.

HLA-A2 transgenic mice are a useful mouse model to translate vaccine studies from mice to humans. These mice express a chimeric class I molecule consisting of α1 and α2 domains of HLA-A2.1 and the α3 transmembrane and cytoplasmic domains of $H\text{-}2K^b$. Using the influenza virus infection model, it was shown that mice generated a similar CTL response to that seen in an influenza-specific A2.1-restricted CTL clone from humans [28]. Hence, the α1 and α2 domains play a dominant role in deciding the CTL specific repertoire and thus, they are used to identify HLA-A2 restricted CD8 T cell epitopes of pathogens [38] and tumors [39]. In addition, they are useful to determine the efficacy of vaccines which are clinically relevant such as the measurement of HLA-A2 restricted CTL responses [40,41]. We had previously identified the MUC1 T cell epitope in HLA-A2 mice [35]. Here, using HLA-A2 transgenic mice, we further demonstrate that OMPLL-MUC1 DNA and RMPLL-MUC1 DNA immunization induces $CD8^+$ T cell responses against STAPPAHGV peptide, while MUC1 DNA alone was insufficient in inducing such responses.

Many previous MUC1 DNA immunization studies were performed on non-transgenic mouse strains [24,42–45], however the homology between mouse and human MUC1 in the VNTR domain is only 34% [46,47], hence immunizing with human MUC1 DNA would be equivalent of immunizing with a foreign antigen in mice. To more closely mimic the situation of human immunization, experiments were performed in human MUC1 transgenic mice. These mice are unable to reject MUC1-expressing tumor cells unlike wild-type mice [48]. In addition, they fail to induce immunoglobulin class switching to IgG subtypes, indicating T-cell tolerance to MUC1 [48]. In this study, although MUC1 DNA alone immunization was able to induce T cell responses against MUC1 in C57BL/6 mice, it was however not able to induce a significant response in MUC1 transgenic mice. Hence, immunization with DNA alone may be insufficient to break tolerance in clinical settings. In contrast, OMPLL-MUC1

DNA and RMPLL-MUC1 DNA immunization was able to break tolerance in MUC1 transgenic mice and induced significant levels of IFN-γ secreting T cells. These results mirror the first MUC1 DNA immunization studies in MUC1 transgenic mice, which showed ID injection of MUC1 DNA alone (of up to 100 μg dose) was insufficient to induce tumor protection against MC38/MUC1 tumor challenge in MUC1 transgenic mice. When MUC1 DNA was co-injected with IL-18 plasmids was the tolerance broken and resulting in protection from subcutaneous challenge [26] and pulmonary metastasis of MC38-MUC1 cells [49]. Hence, the inclusion of pro-inflammatory cytokines plays a role in enhancing the immunogenicity of DNA vaccines and enable tolerance to be broken. In this way, mannan was shown to be able to prime immune responses by inducing DC maturation and cytokine secretion [50], which could be responsible for the immune augmenting properties of OMPLL and RMPLL observed in this study. Preliminary studies on the mechanism of action of OMPLL and RMPLL conjugates revealed an involvement of stimulation of Th1 and Th2 cytokine secretion and TLR activation (manuscript in preparation).

In conclusion, OMPLL-MUC1 DNA and RMPLL-MUC1 DNA are able to induce enhanced MUC1 specific immune responses *in vivo* compared to MUC1 DNA alone in C57BL/6, HLA-A2 and MUC1 transgenic mice. Furthermore, tumor protection is noted at 5 μg and 0.5 μg immunization doses. MUC1 fusion protein linked to OM have been produced under good laboratory and good manufacturing practice conditions and used in clinical trials [17]. The methodology can quite easily be adapted for OMPLL and RMPLL conjugates and subsequent interaction with DNA. In conclusion, OMPLL and RMPLL are effective non-viral ligands for use in DNA-based vaccines in preclinical settings and may poise as a promising vaccine candidate for humans.

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