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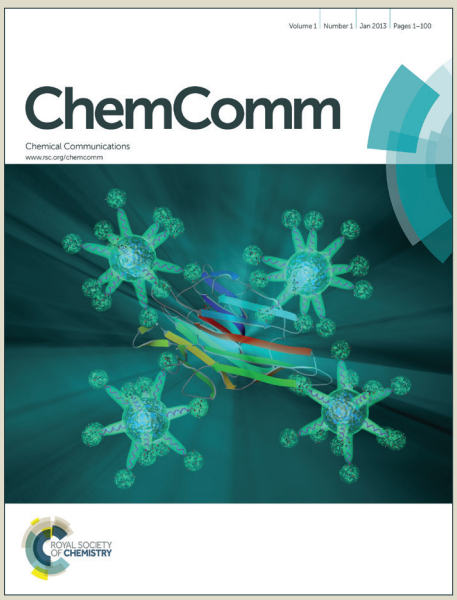

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Synthesis and Immunological Evaluation of Self-Adjuvanting MUC1-Macrophage Activating Lipopeptide 2 Conjugate Vaccine Candidates

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We describe herein the synthesis and immunological evaluation of self-adjuvanting mucin 1 (MUC1)-macrophage activating lipopeptide 2 (MALP2) (glyco)peptide vaccine candidates. Vaccine constructs were shown to induce high titres of class-switched IgG antibodies in C57BL/6 mice after four immunisations despite the lack of a helper T cell epitope.

Mucin 1 (MUC1) is a transmembrane glycoprotein that is normally expressed on the basal membrane of epithelial cells and is known to be highly over-expressed on epithelial tumour cells.[1] The extracellular domain of MUC1 consists of a 20 amino acid variable-number tandem repeat (VNTR) domain that possesses five potential sites of *O*-glycosylation.[2] During tumour progression, alteration in the expression levels of glycosyltransferase enzymes leads to aberrant glycosylation patterns on MUC1 (and other proteins).[3] The result is the presentation of truncated *O*-glycan structures (appended to the side chain of serine (Ser) and threonine (Thr) residues) that are unique to cancer cells and are collectively termed tumour-associated carbohydrate antigens (TACAs).[4] Examples include the monosaccharide Tn (GalNAc-α-Ser/Thr) and disaccharide T (Gal(β1→3)GalNAc-α-Ser/Thr) TACAs. Since the TACA-bearing MUC1 VNTR domains are known to be highly over-expressed in over 90 % of tumours and are not expressed in healthy tissue, they have been widely investigated for use in cancer immunotherapy as vaccine antigens.[5] Importantly, this approach has been validated by the fact that MUC1-based vaccines have entered clinical trials for the treatment of epithelial carcinomas of the breast, colon, pancreas, and lung, among others,[6] and MUC1 was ranked second out of 75 as a candidate antigen for cancer vaccine development.[7] Recently, a number of laboratories have focussed on the development of multi-component vaccines to induce strong immune responses to MUC1 tumour-associated glycopeptides.[8] In recent work, we and others have demonstrated that fully synthetic vaccines possessing the toll-like receptor (TLR) 2/TLR1 agonist Pam_3Cys can elicit a robust immune response without the use of an external adjuvant such as complete Freund's adjuvant (CFA)/incomplete Freund's adjuvant (IFA) which is commonly employed in animal studies but not clinically approved.[9] These studies have incorporated a range of helper T cell (T_h) epitopes to promote the $CD4^+$ T cell responses required to induce antibody isotype class switching. In this study, we were interested in investigating self-adjuvanting MUC1 vaccine candidates possessing macrophage activating lipopeptide 2 (MALP2) as an immunoadjuvant. MALP2 is a lipopeptide derived from *Mycoplasma fermentans* and is a potent activator of Toll-like receptor 2 and 6 heterodimers in dendritic cells, macrophages and B cells.[10] The activation of TLR2/6 leads to the downstream production of pro-inflammatory cytokines TNFα, IL-1, and IL-6, and chemokines MIP-1, MCP-1, IL-8 and RANTES.[11] MALP2 has attracted significant interest as a novel and efficacious immunoadjuvant in infectious disease vaccine development[12] and displays direct anti-tumour activity through inflammation-associated pathways.[13] However, to the best of our knowledge, MALP2 has not been investigated in the context of tumour vaccination. Importantly, it has been demonstrated that B cell stimulation by MALP2 occurs without the need for accessory cells.[10b] We hypothesised that potent, direct stimulation of B cells with MALP2 would induce specific antibody responses without the need for external T_h epitopes. To this end, we set out to test this hypothesis through the synthesis and immunological evaluation of three self-adjuvanting MUC1-MALP2 conjugate vaccine candidates **1-3**. These vaccines differed in the glycosylation state of the MUC1 VNTR and were covalently linked to the MALP2 adjuvant component via a non-immunogenic triethyleneglycolate spacer (Figure 1).

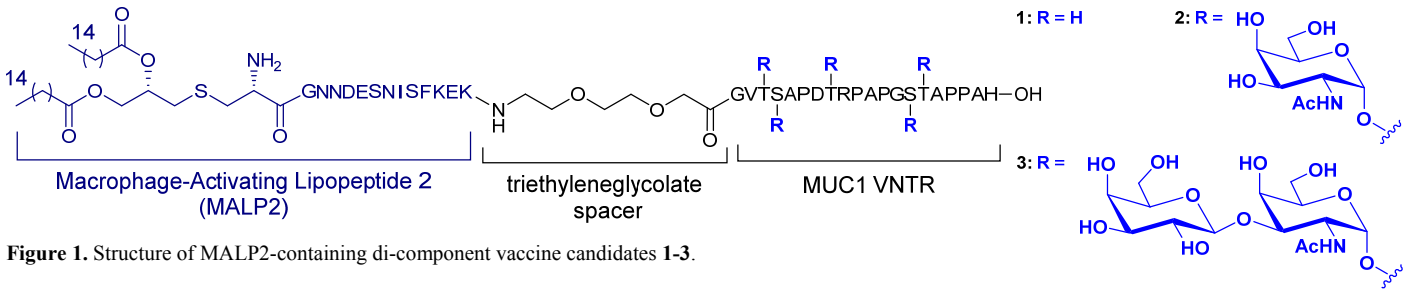
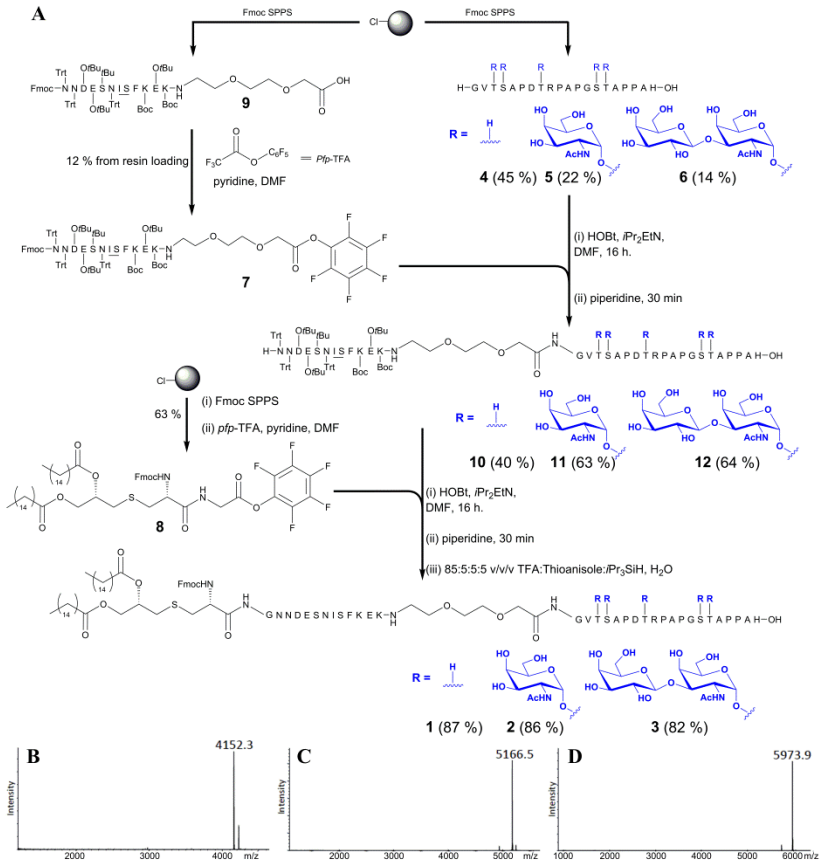


Figure 1. Structure of MALP2-containing di-component vaccine candidates **1-3**.



Scheme 1. A: Synthesis of vaccine candidates **1-3** from fragments **4-7** and **8**. IS = Ile-Ser($\psi^{Me,Me}$Pro) pseudoproline residue. See ESI for detailed conditions.
B-D: MALDI-TOF mass spectra of vaccine candidates **1-3**.

We envisaged that the vaccine constructs containing MALP2 and a single copy of the unglycosylated MUC1 VNTR or the Tn- and T-perglycosylated variants **1-3** could be readily obtained through iterative condensation reactions using three fragments, each readily synthesised by standard Fmoc-strategy solid-phase peptide synthesis (Fmoc-SPPS, Scheme 1). These included the fully deprotected MUC1 VNTR (glyco)peptides **4-6**, the side chain and N-terminally protected peptide fragment of MALP2 containing a triethyleneglycolate spacer that was activated at the C terminus as a pentafluorophenyl (*Pfp*) ester **(7)**, and finally Fmoc-protected Pam_2CysGly-O*Pfp* **(8)** (Scheme 1) (See ESI for synthetic details). (Glyco)peptide fragments **4-6** were synthesised on 2-chlorotrityl chloride (2-Cl-Trt-Cl) resin. For the synthesis of **5** and **6**, peracetylated glycosylamino acid building blocks, corresponding to suitably masked variants of the Tn and T antigen-derived amino acids, were incorporated at the Ser and Thr sites within the MUC1 VNTR sequence using conditions reported previously (see ESI).[9b] Following cleavage from the resin with global side chain deprotection, de-*O*-acetylation of the carbohydrate moieties and purification by HPLC provided **5** and **6** in good yields over the iterative steps. The MALP2 peptide fragment was also synthesised by Fmoc-SPPS on 2-Cl-Trt-Cl resin and incorporated a Ile-Ser($\psi^{Me,Me}$Pro) pseudoproline dipeptide moiety in order to prevent on-resin aggregation and improve the yield of the peptide synthesis. Liberation of the peptide from the solid support was effected *via* treatment with hexafluoroisopropanol (HFIP) in dichloromethane to afford *N*-Fmoc- and side chain-protected peptide **9**. Treatment of crude **9** with pentafluorophenyl trifluoroacetate and pyridine provided protected peptidyl *Pfp*-ester **7** in 12 % isolated yield following purification by normal-phase HPLC. Similarly, Fmoc-Pam_2CysGly-OH was obtained by coupling synthetic Fmoc-Pam_2Cys-OH to Gly immobilised on 2-Cl-Trt-Cl resin followed by cleavage with HFIP. The crude lipopeptide was subsequently converted to the C-terminal *Pfp*-ester using the conditions described above. Following normal-phase HPLC purification, **8** was afforded in 63 % yield. The fragments were next assembled through pentafluorophenyl ester-mediated fragment condensation chemistry[14] to afford the target vaccines **1-3**. Specifically, (glyco)peptides **4-6** were first reacted with *Pfp* ester **7** in the presence of 1-hydroxybenzotriazole (HOBt) and *N,N*-diisopropylethlamine (DIEA) followed by deprotection of the N-Fmoc carbamate *in situ* to provide partially protected conjugates **10-12** in moderate to good yields following purification by semi-preparative C4 HPLC. Finally, **10-12** were reacted with *Pfp*-ester **8** in the presence of HOBt and DIEA. *N*-Fmoc group, followed by cleavage of the side chain protecting groups on the MALP-2 peptide fragment through treatment with an acidic cocktail provided the desired conjugate vaccine targets **1-3** in excellent yields (82-87 %) following purification by semi-preparative C4 HPLC.

In order to investigate the immunological response to the self-adjuvanting MALP2 vaccine candidates, C57BL/6 mice were immunised weekly via sub-cutaneous injections of each vaccine candidate (or PBS as a control). MUC1-specific serum antibodies were enumerated by ELISA throughout the four week immunisation course. Immunised mice exhibited high titres of MUC1-specific IgM antibodies after the first injection which were sustained throughout the immunisation schedule. In addition, high levels of class-switched IgG1, IgG2b and IgG3 antibodies were elicited, titres of which increased after each immunisation. In all cases, mice immunised with the unglycosylated vaccine candidate **1** exhibited the highest specific antibody titres (Fig. 2). Antibodies isolated from all mice were capable of recognising and binding to the MUC1 VNTR (glyco)peptide epitopes against which they were raised. These interactions were selective, with the exception of antibodies raised against the Tn-containing vaccine, which also reacted with unglycosylated and T antigen-containing MUC1 VNTR (glyco)peptides, as determined by ELISA, (see ESI). This cross-reactivity of antibodies raised against Tn-containing MUC1 vaccines to unglycosylated and T-containing MUC1 (glyco)peptide antigens mirrors observations by Clausen and co-workers.[2] Humoral immunity to MUC1 is considered a key factor controlling the growth and metastasis of human cancer. The ability of MALP2 vaccines **1-3** to promote robust IgG1, IgG2b and IgG3 MUC1-specific antibodies is likely to be particularly important given their role in mediating antibody-dependent cell-mediated cytotoxicity (ADCC) and complement activation.[15] $CD8^+$ and $CD4^+$ T cell responses were examined by *in vivo* CTL assay and *in vitro* cytokine staining, but specific cytotoxic activity against glycoforms of the H-$2K^b$-binding epitope SAPDT*RPAP[16] was not observed (See ESI). In addition, there was no increase in IL-4, IFN-γ or CD25-expressing $CD4^+$ or $CD8^+$ T cells compared to PBS-treated controls (See ESI). This lack of observed T_h response, together with the sustained high levels of IgM observed,[17] leads us to propose that these vaccines induced T cell-independent class switching. Future experiments involving CD4-depleted mice will explore this hypothesis further.

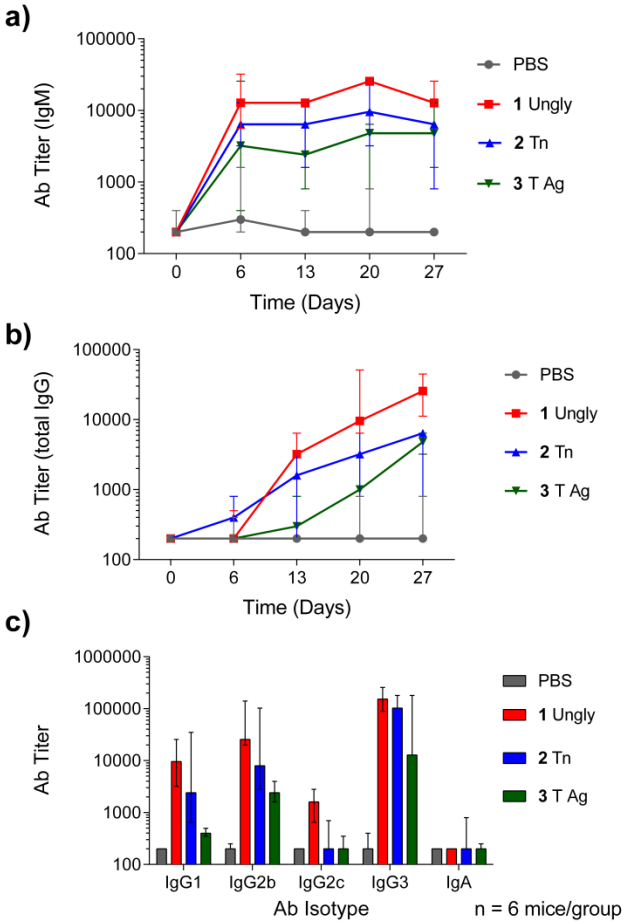


Figure 2: a), b) Total MUC1-specific titres of **a)** IgM and **b)** IgG over time. **c)** Day 27 IgG isotype titres from the sera of mice immunised with PBS or vaccine candidates **1-3**. Mice were immunised on days 1, 7, 14, and 21 of the experiment. Plotted points represent median (± interquartile range) endpoint titres of n = 6 C57BL/6 mice. See ESI for experimental conditions.

Conclusions

In summary, we have successfully synthesised a number of (glyco)lipopeptide self-adjuvanting MUC1-MALP2 conjugate vaccine candidates. These self-adjuvanting vaccine candidates induced robust humoral immune responses in animal models with class switched antibodies of several isotypes, indicative of poly-functional humoral immune responses. Importantly, this response occurred in the absence of an external adjuvant or helper T cell epitope. Future work in our laboratory will involve the use of MALP2 in conjunction with a T_h epitope to investigate the role of T cell help in immune responses to MALP2 vaccines.

Notes and references

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Electronic Supplementary Information (ESI) available: Full synthetic procedures, characterisation of novel compounds and immunological methods and data. See DOI: 10.1039/c000000x/

1. M. A. Tarp and H. Clausen, *Biochim. Biophys. Acta*, 2008, **1780**, 546-563.
2. M. A. Tarp, A. L. Sorensen, U. Mandel, H. Paulsen, J. Burchell, J. Taylor-Papadimitriou and H. Clausen, *Glycobiology*, 2007, **17**, 197-209.
3. a) E. Meezan, H. C. Wu, P. H. Black and P. W. Robbins, *Biochemistry*, 1969, **8**, 2518-2524; b) J. W. Dennis, M. Granovsky and C. E. Warren, *Biochim. Biophys. Acta*, 1999, **1473**, 21-34.
4. a) S. Hakomori, *Adv. Cancer Res.*, 1989, **52**, 257-331; b) D. S. Sanders and M. A. Kerr, *Mol. Pathol.*, 1999, **52**, 174-178.
5. a) T. Buskas, P. Thompson and G. J. Boons, *Chem. Commun.*, 2009, 5335-5349; b) P. Beatty, S. Ranganathan and O. J. Finn, *Oncoimmunology*, 2012, **1**, 263-270; c) N. Gaidzik, U. Westerlind and H. Kunz, *Chem. Soc. Rev.*, 2013, **42**, 4421-4442.
6. C. K. Tang, M. Katsara and V. Apostolopoulos, *Expert Rev. Vaccines*, 2008, **7**, 963-975.
7. M. A. Cheever, J. P. Allison, A. S. Ferris, O. J. Finn, B. M. Hastings, T. T. Hecht, I. Mellman, S. A. Prindiville, J. L. Viner, L. M. Weiner and L. M. Matrisian, *Clin. Cancer Res.*, 2009, **15**, 5323-5337.
8. a) A. Hoffmann-Roder, A. Kaiser, S. Wagner, N. Gaidzik, D. Kowalczyk, U. Westerlind, B. Gerlitzki, E. Schmitt and H. Kunz, *Angew. Chem. Int. Ed.* , 2010, **49**, 8498-8503; b) N. Gaidzik, A. Kaiser, D. Kowalczyk, U. Westerlind, B. Gerlitzki, H. P. Sinn, E. Schmitt and H. Kunz, *Angew. Chem. Int. Ed.* , 2011, **50**, 9977-9981; c) H. Cai, Z. H. Huang, L. Shi, Z. Y. Sun, Y. F. Zhao, H. Kunz and Y. M. Li, *Angew. Chem. Int. Ed.*, 2012, **51**, 1719-1723.
9. a) S. Ingale, M. A. Wolfert, J. Gaekwad, T. Buskas and G. J. Boons, *Nat. Chem. Biol.*, 2007, **3**, 663-667; b) B. L. Wilkinson, S. Day, L. R. Malins, V. Apostolopoulos and R. J. Payne, *Angew. Chem. Int. Ed.* , 2011, **50**, 1635-1639; c) V. Lakshminarayanan, P. Thompson, M. A. Wolfert, T. Buskas, J. M. Bradley, L. B. Pathangey, C. S. Madsen, P. A. Cohen, S. J. Gendler and G. J. Boons, *Proc. Natl. Acad. Sci. U.S.A.*, 2012, **109**, 261-266; d) B. L. Wilkinson, S. Day, R. Chapman, S. Perrier, V. Apostolopoulos and R. J. Payne, *Chem. Eur. J.*, 2012, **18**, 16540-16548.
10. a) P. F. Muhlradt, M. Kiess, H. Meyer, R. Sussmuth and G. Jung, *J. Exp. Med.*, 1997, **185**, 1951-1958; b) S. Borsutzky, K. Kretschmer, P. D. Becker, P. F. Muhlradt, C. J. Kirschning, S. Weiss and C. A. Guzman, *J. Immunol.*, 2005, **174**, 6308-6313.
11. a) P. F. Muhlradt and U. Schade, *Infect. Immun.*, 1991, **59**, 3969-3974; b) U. Deiters and P. F. Muhlradt, *Infect. Immun.*, 1999, **67**, 3390-3398.
12. F. Rharbaoui, B. Drabner, S. Borsutzky, U. Winckler, M. Morr, B. Ensoli, P. F. Muhlradt and C. A. Guzman, *Eur. J. Immunol.*, 2002, **32**, 2857-2865.
13. C. Schneider, T. Schmidt, C. Ziske, K. Tiemann, K. M. Lee, V. Uhlinsky, P. Behrens, T. Sauerbruch, I. G. Schmidt-Wolf, P. F. Muhlradt, J. Schmidt and A. Marten, *Gut*, 2004, **53**, 355-361.
14. B. L. Wilkinson, L. R. Malins, C. K. Chun and R. J. Payne, *Chem. Commun.*, 2010, **46**, 6249-6251.
15. a) C. I. Bindon, G. Hale, M. Bruggemann and H. Waldmann, *J. Exp. Med.*, 1988, **168**, 127-142; b) S. Von Mensdorff-Pouilly, M. Moreno and R. H. Verheijen, *Cancers*, 2011, **3**, 3073-3103.
16. V. Apostolopoulos, J. S. Haurum and I. F. McKenzie, *Eur. J. Immunol.*, 1997, **27**, 2579-2587.
17. J. J. Mond, A. Lees, C. M. Snapper, *Annu. Rev. Immunol.*, 2005, **13**, 655–692.