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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 Pam₃Cys 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 (T₉) 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 TNFa, 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 development¹² 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 T₉ 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-glycosylation state of the MUC1 VNTR and were covalently linked conjugate vaccine candidates. Three vaccines differed in the glycosylation state of the MUC1 VNTR and were covalently linked conjugate vaccine candidates. These vaccines differed in the glycosylation state of the MUC1 VNTR and were covalently linked conjugate vaccine candidates.

Figure 1. Structure of MALP2-containing di-component 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 triethylene glycolate spacer that was activated at the C terminus as a peracetylated glycosylamino acid building block, 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).70 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(ψMe,MePro) pseudoproline dipeptide moiety in order to prevent cis-dimerisation 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.7 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.13 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-2Kb-binding epitope SAPDTRPAP16 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 Th1 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.

For the synthesis of MALP2 we envisaged that the iterative condensation reactions using three fragments, each readily synthesised by standard Fmoc-strategy solid-phase peptide synthesis (Fmoc-SPPS, Scheme 1). One of these included the fully deprotected MALP2 peptide 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). One of these included the fully deprotected MALP2 peptide 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). One of these included the fully deprotected MALP2 peptide 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). One of these included the fully deprotected MALP2 peptide 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). One of these included the fully deprotected MALP2 peptide 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). One of these included the fully deprotected MALP2 peptide 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). One of these included the fully deprotected MALP2 peptide 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).
involve the use of MALP2 in conjunction with a Th epitope to induce robust humoral immune responses in animal models. Future work in our laboratory will investigate the role of T cell help in immune responses to MALP2 vaccines.

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<sub>h</sub> epitope to investigate the role of T cell help in immune responses to MALP2 vaccines.

Notes and references


