Recombinant plant-expressed tumour-associated MUC1 peptide is immunogenic and capable of breaking tolerance in MUC1.Tg mice

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Summary

The human epithelial mucin MUC1 is a heavily glycosylated transmembrane protein that is overexpressed and aberrantly glycosylated on over 90% of human breast cancers. The altered glycosylation of MUC1 reveals an immunodominant peptide along its tandem repeat (TR) that has been used as a target for tumour immunotherapy. In this study, we used the MUC1 TR peptide as a test antigen to determine whether a plant-expressed human tumour-associated antigen can be successfully expressed in a plant system and whether it will be able to break self-antigen tolerance in a MUC1-tolerant mouse model. We report the expression of MUC1 TR peptide fused to the mucosal-targeting *Escherichia coli* enterotoxin B subunit (LTB-MUC1) in a plant host. Utilizing a rapid viral replicon transient expression system, we obtained high yields of LTB-MUC1. Importantly, the LTB-MUC1 fusion protein displayed post-translational modifications that affected its antigenicity. Glycan analysis revealed that LTB-MUC1 was glycosylated and a MUC1-specific monoclonal antibody detected only the glycosylated forms. A thorough saccharide analysis revealed that the glycans are tri-arabinans linked to hydroxy-prolines within the MUC1 tandem repeat sequence. We immunized MUC1-tolerant mice (MUC1.Tg) with transiently expressed LTB-MUC1, and observed production of anti-MUC1 serum antibodies, indicating breach of tolerance. The results indicate that a plant-derived human tumour-associated antigen is equivalent to the human antigen in the context of immune recognition.

Introduction

The human epithelial mucin MUC1 is a heavily glycosylated transmembrane protein that is expressed on simple secretory epithelial cells of ducts and glands. MUC1 is overexpressed and aberrantly glycosylated on many human epithelial tumours, including more than 90% of human breast cancers (Mukherjee *et al.*, 2003). The altered glycosylation of MUC1 on tumour cells reveals an immunodominant peptide sequence along its tandem repeat (TR) that has been used as a target for tumour immunotherapy in a number of preclinical and clinical studies (Acres *et al.*, 1993, 2000; Ding *et al.*, 1993; Apostolopoulos *et al.*, 1994; Scholl *et al.*, 2000; Mukherjee *et al.*, 2001, 2003, 2004; Rochlitz *et al.*, 2003). On normal epithelial cells, MUC1 is polarized and restricted to the apical surface of the cell, whereas on tumour cells MUC1 is no longer restricted and is found throughout the tumour mass. In breast cancer, MUC1 expression correlates with high metastatic potential and poor survival (Nakamori *et al.*, 1994; McGuckin *et al.*, 1995; Leroy *et al.*, 2002). The 2009 Cancer Statistics report noted that cancers expressing aberrant MUC1 accounted for 72% of new cases and 66% of the deaths (Jemal *et al.*, 2009). The altered expression and glycosylation pattern of MUC1 on tumour cells makes it an attractive target for cancer immunotherapy, especially with the recent finding that the TR of MUC1 is a target for cytotoxic T lymphocytes (CTLs; Jerome *et al.*, 1991; Ioannides *et al.*, 1993; Reddish *et al.*, 1998). Importantly, the lymphocytes that target the aberrant form of MUC1 do not recognize the nonaberrant form on healthy cells and therefore do not cause autoimmunity. However, like many other tumour-associated antigens, MUC1 is a self-antigen that is expressed on secretory epithelia neonatally and postnatally; thus, the T- and B-cell repertoires are immunologically tolerant to MUC1. To evaluate vaccines directed against MUC1 as a self-antigen, a transgenic mouse model that expresses the human MUC1 molecule on an inbred C57BL/6 background (MUC1.Tg) was created. The MUC1.Tg mice carry the human MUC1 transgene driven by its own promoter and express MUC1 in a developmentally regulated and tissue-specific fashion similar to humans (Rowse *et al.*, 1998).

The use of plants for the production and delivery of vaccine proteins is emerging as an attractive alternative to traditional production systems (Tacket, 2005). Producing vaccines in plants reduces the risk of contamination with animal pathogens and offers the possibility for large-scale production at potentially low costs. Vaccine antigens can be produced in plants via stable genetic transformation or by virus-based transient expression systems. Stable genetic transformation system involves

integrating the gene of interest into the plant genome and allows the successive propagation of the transgenic line via seeds or vegetative cuttings. Transient expression systems often use a recombinant virus that carries the gene of interest and which upon infection causes the plant to express the vaccine antigen. Typically, transient expression produces substantially higher expression than stable transgenic plants. Specifically, a deconstructed viral vector system known as magnICON™ (Icon Genetics, Halle, Germany) is based on *in planta* assembly of functional viral replicons from separate pro-vector modules (Marillonnet *et al.*, 2004). With this system, *Agrobacterium* cells are used to deliver various modules that are assembled inside the cell with the help of a site-specific recombinase (integrase). The resulting DNA is transcribed and spliced to remove the recombination sites, thus generating a fully functional replicon. With the magnICON™ expression system, GFP expression in leaves of *Nicotiana benthamiana* was reported at 80% total soluble protein (TSP; Marillonnet *et al.*, 2004) compared to the <1% TSP achieved with stable transgenic plants. The magnICON™ system is rapid, generating high expression in 7–14 days postinfiltration, compared to the months required for stable plant transformation.

Subunit vaccines consist of one or more proteins that contain the antigenic fingerprint of the disease-causing agent but do not cause disease. They are usually monomeric antigens that are not particulate or conglomerate and are often poor immunogens. Because small molecules with a molecular weight of <2–5 kDa are not usually immunogenic, even when administered in the presence of adjuvant, we decided to fuse the MUC1 TR to a carrier molecule. A bacterial product with great potential to act as a carrier for subunit vaccines is the heat-labile toxin (LT) of enterotoxigenic *Escherichia coli*. LT and the closely related *Vibrio cholerae* cholera toxin (CT) bind to ganglioside receptors on all nucleated cells and have strong immuno-enhancing effects on both systemic and mucosal immune responses (Rappuoli *et al.*, 1999). LT contains a nontoxic B subunit (LTB) and a toxic A subunit (LTA). The B subunit forms a doughnut-shaped pentamer that targets the holotoxin to the gut-associated lymphoid tissue (GALT) by binding to M cells found on the surface of Peyer's patches. The potent mucosal immunogenicity of LT and LTB depends in part on the ability to bind to gangliosides (Guidry *et al.*, 1997; de Haan *et al.*, 1998). Recent evidence suggests that CT specifically binds directly to antigen-presenting cells (APCs) through GM1–ganglioside interaction and induces nuclear translocation of nuclear factor (NF)-kB, leading to DC maturation and activation (Kawamura *et al.*, 2003). Therefore, LTB may not only be useful as a carrier for mucosal immunizations because of their M-cell interaction in the Peyer's patches, but also more generally as an immuno-modulating APC-targeting molecule. Although LTB without LTA exhibits reduced antigenicity (Horner *et al.*, 1998; Bagley *et al.*, 2002, 2003), LTB may be used as an effective carrier by fusing the vaccine antigen to LTB without the toxic A subunit.

In this study, we report the expression of the human tumour-associated antigen MUC1 tandem repeat peptide and its fusion to the mucosal carrier peptide LTB (LTB-MUC1) in a plant expression system. Utilizing the magnICON™-deconstructed viral vector system, we efficiently produced LTB-MUC1 that was post-translationally modified. LTB-MUC1 was glycosylated, and a MUC1-specific monoclonal antibody detected the glycosylated forms of LTB-MUC1, but not the nonglycosylated form. We immunized MUC1.Tg mice with the magnICON™-expressed LTB-MUC1 and showed breach of MUC1 self-antigen tolerance. To our knowledge, this is the first study to report the expression of a human breast cancer tumour-associated antigen in a plant system and its success in breaking self-antigen tolerance in a transgenic mouse model.

Results

Nucleic acid analysis of agroinfiltrated plants

MagnICON™ transient expression was performed with one of the 3′ module constructs pICH-LTB, pICH-LM1 or pICH-LM1His (Figure 1a) co-delivered with the 5′ module pICH15879 and the integrase module pICH14011 (Figure 1a) into *N. benthamiana*
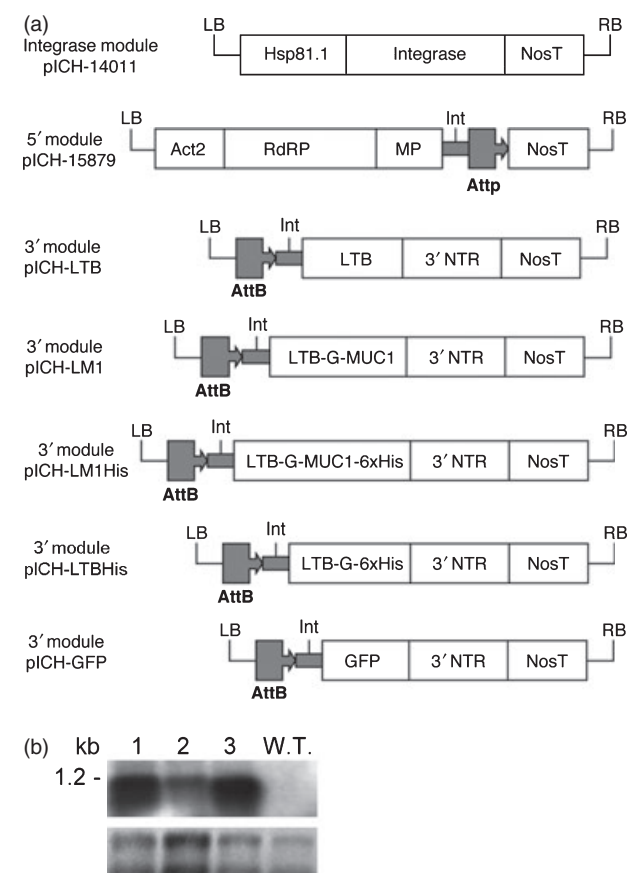
Figure 1 magnICON™ viral vector transient expression system. (a) Diagrammatic representation of magnICON™ pro-vector modules used in this study. Hsp81.1 represents the Arabidopsis heat shock protein 81.1 promoter; Integrase, *Streptomyces* phage PhiC31 integrase; AttP and AttB, PhiC31 integrase recombination sites; Act2, Arabidopsis actin 2 promoter; RdRP, RNA-dependent RNA polymerase; MP, movement protein; LTB, synthetic LTB gene; LTB-G-MUC1, LTB gene with the GPGP linker and the MUC1 tandem repeat; LTB-G-MUC1-6His, LTB-G-MUC1 gene with a histidine tag on the C-terminus of MUC1; GFP, green fluorescent protein; LTBHis, LTB gene with the SGPS linker and a histidine tag, 3′NTR, 3′ untranslated region; NosT, nopaline synthase terminator; Int, intron; LB and RB, left and right borders of the T-DNA region. (b) Northern analysis of *Nicotiana benthamiana* infiltrated with magnICON™ pro-vector modules, for the detection of LTB-MUC1 mRNA. Lanes: 1–2, *N. benthamiana* infiltrated with pICH-LM1; 3, *N. benthamiana* infiltrated with pICH-LTB; W.T., noninfiltrated *N. benthamiana* control. Bottom panel is the methylene blue-stained membrane for loading control: 10 μg of total RNA was loaded for each sample.

leaves. Northern blot analysis for leaves infiltrated with pICH-LM1His (Figure 1b, lanes 1 and 2) exhibits a band at approximately 1.2 kb, while leaves infiltrated with pICH-LTB (lane 3) showed bands that are slightly lower (approximately 1.0 kb). No bands are present in the wild-type control lane. Therefore, the magnICON™ vectors produced correct transcripts with no detectable truncations or degradation products.

LTB-MUC1 expression in agroinfiltrated plants

Nicotiana benthamiana leaves infiltrated with pICH-LM1 were evaluated for LTB-MUC1 expression at different time points [7, 9 and 11 days postinfiltration (dpi)] to determine the optimum harvest time (Figure 2). Slight necrosis was apparent in LTB-MUC1-expressing leaves 9 dpi, with complete necrosis at infiltration sites by day 11 (Figure 2a), yet leaves infiltrated with pICH-GFP remained healthy throughout the 11-day time course. The necrosis indicates that LTB-MUC1 expression was toxic to the leaf tissue, which was observed in another study that expressed hepatitis B core antigen using the magnICON™ system (Huang *et al.*, 2006). We found that expression of unfused LTB (without MUC1) induced earlier and stronger necrosis than LTB-MUC1 (data not shown), which suggests that the toxic effect of LTB-MUC1 is as a result of the LTB moiety.

Labile nontoxic B subunit levels in LTB-MUC1-expressing leaves were 70 μg/g fresh weight (μg/g f.w.) at 7 dpi, dropped to 38 μg/g f.w. on day 9 and then increased on 11 dpi to 88 μg/g f.w. (Figure 2b). MUC1 levels peaked at 7 dpi with 16 μg/g f.w. and then leveled off at 9 and 11 dpi with 12 μg/g f.w. (Figure 2c). The molecular mass ratio between fusion partners LTB and MUC1 is 6 (12-kDa LTB vs. 2-kDa MUC1), which is consistent with the observed lower levels of MUC1 peptide than LTB. We cannot explain the difference in expression level time courses for LTB vs. MUC1, but we suggest that LTB may be more stable during later stages of tissue degradation (Figure 2a). The tissue necrosis is probably due to the hypersensitive response that plants use to combat pathogen infection (Mur *et al.*, 2008). This process involves an oxidative burst and induces a series of biochemical responses, including the release of serine proteases (Antao and Malcata, 2005) which could result in cleavage to remove MUC1 from LTB. Because we used a ganglioside capture ELISA for both LTB and MUC1 detection, a cleavage of MUC1 from LTB would result in the lower levels of MUC1 compared to LTB detected on 11 dpi. From these results, we concluded that 7 dpi was the optimum harvest time for LTB-MUC1 expression with the magnICON™ system.

To facilitate purification of the LTB-MUC1 fusion protein, we fused a 6His-tag to the C-terminus of the MUC1 gene to create the pICH-LM1His pro-vector module. We also constructed the pICH-LTBHis pro-vector module that contained a C-terminal short linker and 6His-tag. The LTB-6His and LTB-MUC1-6His proteins were transiently expressed in *N. benthamiana*, followed by metal affinity purification. Purified proteins were analysed by SDS–PAGE and Western blots for LTB, His and MUC1 (Figure 3a–c, respectively). The anti-LTB probe for LTB-MUC1 showed a protein at the expected 16 kDa size, and two unexpected proteins at approximately 18 and 20 kDa (Figure 3, Lanes LM1). The anti-His and anti-MUC1 probes detected the 18- and 20-kDa proteins (Figure 3b,c), but not the 16-kDa protein. MagnICON™-expressed LTB (Figure 3) showed the expected 12-kDa protein with anti-LTB and anti-His probes, but no 18- or 20-kDa species, and no reaction with the anti-MUC1 probe. These observations indicate that the unexpected 18- and 20-kDa proteins were because of the MUC1 peptide, which may have been post-translationally modified.

Because MUC1 is a highly glycosylated protein in humans and has a total of five Ser or Thr residues in the TR sequence (sites for *O*-glycosylation), we decided to investigate whether the unexpected proteins resulted from glycosylation. *In silico* analyses using NetNGlyc 1.0 predicted no potential *N*-glycosylation sites in our LTB-MUC1 sequence. According to NetOGlyc 3.1, the LTB-MUC1 construct has seven potential mucin type *O*-glycosylation sites. However, a recent study showed that plants do not have the enzymes necessary for mucin type *O*-glycosylation (Daskalova *et al.*, 2010). Thus, we expect that Ser/Thr sites are not utilized in plants, where *O*-glycosylation occurs on hydroxyproline (Hyp) residues. The LTB-MUC1-6His protein contains eight Pro residues with the motifs xPxP and xPPxx, which may be recognized by plant prolyl hydroxylases (Xu *et al.*, 2008).

Hydroxyproline is a site for arabinogalactin type O-glycosylation, in which linear or branched oligosaccharides of arabinose and/or galactose are attached to Hyp (Xu *et al.*, 2008). To perform a thorough glycan analysis for the LTB-MUC1-6His fusion protein, we prepared highly purified proteins. We employed galactose affinity followed by metal affinity, which yielded greater purity than metal affinity alone. The purified proteins were separated by SDS–PAGE (Figure 4a), and excised bands from the gel were dried, hydrolysed for 3 h with 2 M TFA and the released monosaccharides were derivatized with anthranilic acid. The amino acyl-sugars were analysed by RP-HPLC. On this gel (Figure 4a), the LTB-MUC1-6His 20-kDa protein appeared to
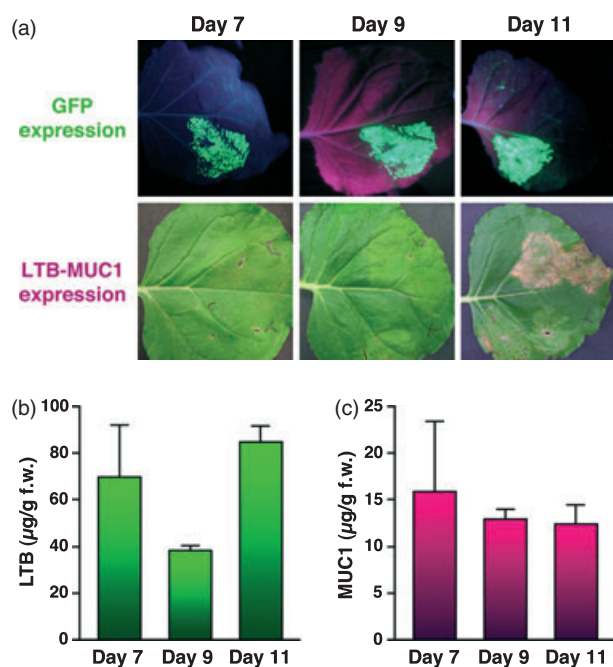
Figure 2 Time course of LTB-MUC1 expression in magnICON™ infiltrated *Nicotiana benthamiana*. (a) Expression of GFP and LTB-MUC1 in *N. benthamiana* 7, 9 and 11 days postinfiltration (dpi). Slight necrosis is apparent in LTB-MUC1-expressing leaves 9 dpi, with complete necrosis at infiltration site by day 11. Ganglioside capture ELISA was used to quantify LTB (b) and MUC1 (c) expression at 7, 9 and 11 days postinfiltration. Expression levels reported in micrograms per gram fresh weight (μg/g f.w.). Data are means of three independent leaf infiltrations ±SD.
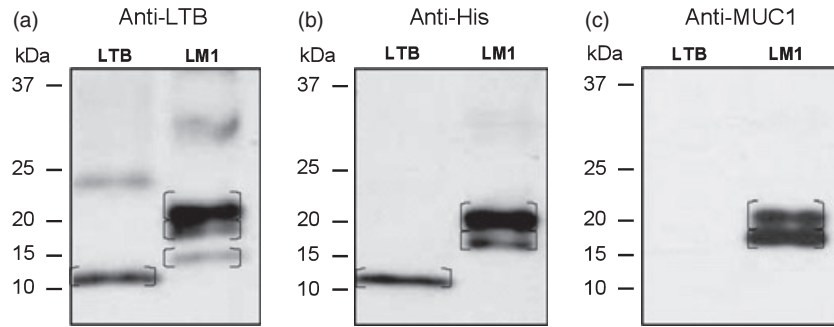
Figure 3 Western blot analysis of metal affinity-purified magnICON™-expressed LTB-6His and LTB-MUC1-6His. (a) Anti-LTB antibody was used to detect LTB-6His (lane LTB) and LTB-MUC1-6His (lane LM1). The LTB lane showed the LTB monomer at the expected molecular mass of 12 kDa as well as a possible dimer at 24 kDa. The LM1 lane showed the expected size of the LTB-MUC1-6His monomer at 16 kDa, as well as two higher molecular mass proteins at approximately 18 and 20 kDa. The protein detected at approximately 32 kDa may represent the dimer for the LTB-MUC1-6His. (b) Nickel-conjugated HRP was used to detect LTB-6His (lane LTB) and LTB-MUC1-6His (lane LM1). The LTB lane showed the His-tagged LTB monomer at 12 kDa. The LM1 lanes only showed the higher molecular mass LTB-MUC1-6His proteins, approximately 18 and 20 kDa, but not the 16-kDa protein. (c) Anti-MUC1 BC2 monoclonal antibody was used to detect LTB-6His (lane LTB) and LTB-MUC1-6His (lane LM1). No MUC1 protein was detected in the LTB lane. Only the approximately 18 and 20 kDa of LTB-MUC1-6His were detected by the MUC1-specific antibody.
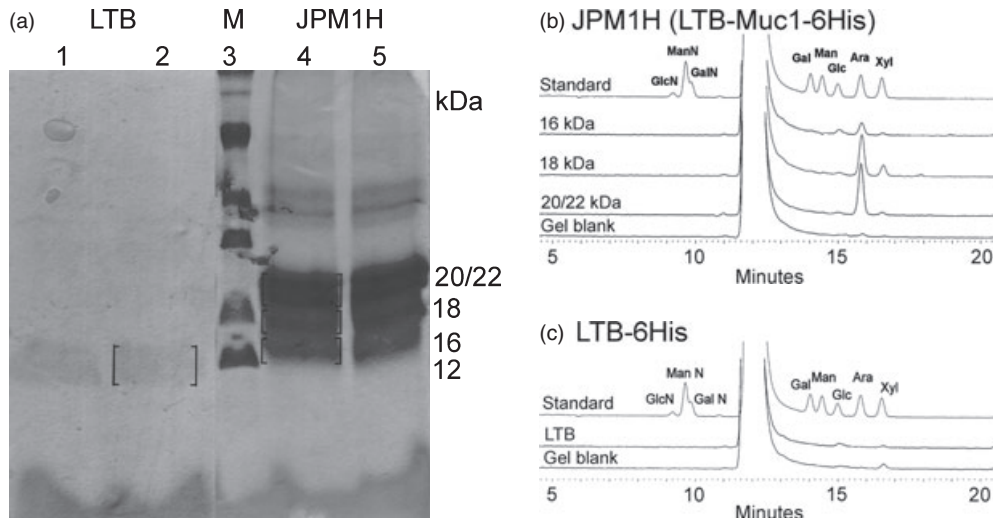
Figure 4 Glycosylation analysis of LTB-Muc1-6His. (a) Coomassie-stained SDS–PAGE gel of purified LTB-6His (lanes 1 and 2) and LTB-MUC1-6His (lanes 4 and 5) protein fractions. Gel bands at approximately 12 kDa (LTB-6His) and 16, 18 and 20/22 kDa (LTB-Muc1-6His) were excised and hydrolysed with TFA to release monosaccharides. (b) RP-HPLC of LTB-MUC1-6His shows a distinct peak for arabinose from the 20/22 and 18-kDa molecular weight fractions, and a shorter peak from the 16-kDa fraction. An excised piece from the gel served as a negative control (Gel blank). (c) RP-HPLC of LTB-6His does not show any monosaccharide peaks, suggesting the LTB is not glycosylated. The standard contained the following glycans: GlcN, Glucosamine; ManN, Mannosamine; GalN, Galactosamine; Gal, Galactose; Man, Mannose; Glc, Glucose; Ara, Arabinose; Xyl, Xylose. Gel blank was used as a negative control.

resolve a doublet, which were sampled together and referred to as a 20/22-kDa band. Overlaid HPLC chromatograms of AA-sugars from gel bands (16, 18 and 20/22 kDa) revealed that the higher molecular weight proteins (18 and 20/22 kDa) contained arabinose, whereas the lower molecular weight band (16 kDa) contained much less sugar (Figure 4b, Table 1). No sugars were present on LTB-6His peptide (Figure 4c, Table 1). As depicted in Table 1, the 20/22-kDa protein contained the highest amount of arabinose (2.19 nmol); the 18-kDa protein contained a lower amount (1.70 nmol); the 16-kDa protein contained the least amount of arabinose (0.44 nmol).

Fractions from LTB-MUC1-6His (sequence shown in Figure 5a) were pooled and subjected to pronase digestion. An MS/MS spectrum of the glycopeptide GSTAOOAHG + 3 Ara, where O designates Hyp, shows that the APP sequence contains hydroxyproline and is furnished with a tri-arabinan (Figure 5b). Pronase digestion of LTB-6His did not reveal any glycopeptides. Pooled fractions of LTB-MUC1-6His were then subjected to alkaline degradation with Ba(OH)$_2$. The resulting amino acids and glyco-amino acids were dansylated and analysed by carbon-LC-ESI-MS. The LC-MS result for LTB-MUC1-6His (Figure 6a) showed Hyp (33%) and Ara-3Hyp (11%), which eluted as isomeric peaks, while Pro (55%) eluted as a single peak. LTB-6His did not show any arabinose residues, although there were a number of hydroxylated prolines (26%, Figure 6b).

Although pronase digestion revealed that the prolines (**PP**) within the MUC1 sequence GSTA***PP***AHG are hydroxylated and

Table 1 Arabinose detected in each protein fraction of LTB-MUC1-6His and LTB-6His

Sample	Gel band	nmol Arabinose/band
LTBMUC1	20–22 kDa	2.19
	18 kDa	1.70
	16 kDa	0.44
	Gel blank	0.00
LTB	14 kDa	0.00
	Gel blank	0.00

furnished with a tri-arabinan (Figure 5b), we note that there is another GSTAPPA sequence at the C-terminal end near the histidine tag, which also occurs in a context for O-linked hydroxyproline arabinosylation, but was not detected by pronase digestion. Interestingly, Hyp-Ara(3) modification to LTB-MUC1-6His would only add approximately 0.5 kDa per site, which is substantially less than the observed mobility shifts. However, it is possible that carbohydrates may cause rather unexpected effects in SDS–PAGE because of the nonbinding of SDS. Another characteristic trait of glycosylated protein is the heterogeneity in mobility, which results in broad bands in all the Western blots of LTB-MUC1 compared to LTB alone. Thus, although we cannot fully explain the mobility shifts of the glycosylated species, we clearly showed that LTB-MUC1 protein is glycosylated by O-linked hydroxyproline arabinosylation.

Systemic immunogenicity of purified LTB-MUC1-6His in MUC1.Tg mice

As our main interest was to see whether a plant-derived human cancer-associated antigen can mimic the authentic peptide and be utilized as a vaccine candidate, we parenterally delivered the LTB-MUC1-6His fusion protein to determine whether it could break self-antigen tolerance and induce a MUC1-specific response in a tolerant mouse model. We injected the plant-derived LTB-MUC1-6His protein into MUC1.Tg mice. Because we previously observed that the addition of CpG ODN to a vaccine formulation decreases the amount of antigen necessary to induce an immune response (Pinkhasov et al., 2010), we also tested the addition of 100 μg CpG ODN to the plant-derived LTB-MUC1-6His (2–20 μg). The MUC1.Tg mice were immunized on days 0, 7, 14 and 35. Serum analysis for anti-LTB IgG (data not shown) and anti-MUC1 IgG (Figure 7a) showed responses after the second immunization and a robust response after the boost on day 35 for the CpG ODN (100 μg) + LTB-MUC1-6His treatment group. Analysis of anti-MUC1 IgG1 and IgG2a showed that there was a Th2 bias without CpG ODN (indicated by a relatively high IgG1; no IgG2a detected in the no CpG group; Figure 7b,c). However, addition of CpG ODN to LTB-MUC1-6His pushed the response towards Th1 response, as shown by substantial IgG2a production (Figure 7c). Anti-MUC1 antibody titres (IgG, IgG1 and IgG2a) in mice treated with CpG ODN + LTB-MUC1 are significantly higher ($P < 0.05$) than the PBS control group.

Discussion

Over the past 15 years, plants have become a promising alternative for producing recombinant proteins and potential therapeutics (Haq et al., 1995; Fischer et al., 1999; Mason et al., 2002). In addition to the potential low cost, biological safety and large-scale production advantage, plants are higher eukaryotic organisms with an endomembrane system. Therefore, they fold and assemble complex proteins using chaperones and perform post-translational modifications similar to mammalian systems (Ma et al., 2005). In this study, we investigated whether the tumour antigen MUC1 fused to the mucosal-targeting peptide LTB could be properly expressed in a plant system. We employed a novel deconstructed viral vector system known as magnICON^{TM} (Marillonnet et al., 2004, 2005; Gleba et al., 2005).
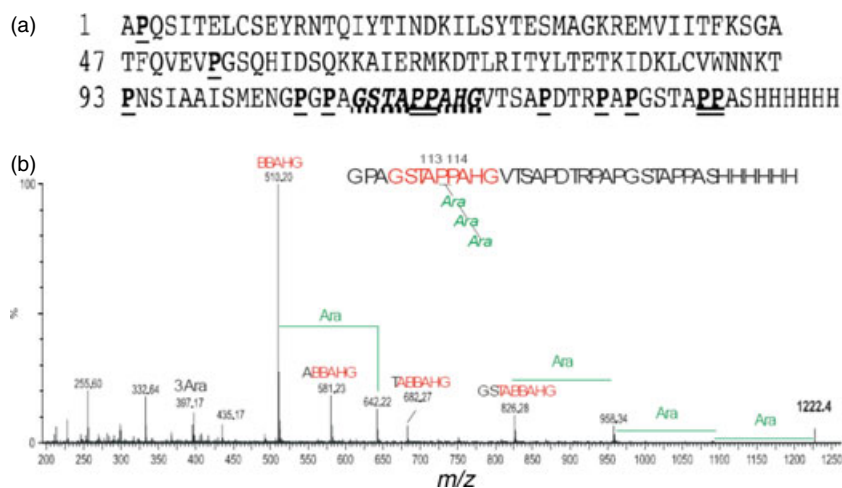
Figure 5 Pronase peptide analysis of LTB-MUC1-6His by MS and MS/MS. (a) Amino acid sequence of LTB-MUC1-6His. Numbers at left indicate the amino acid positions from the N-terminus. The amino acid sequence GSTAPPAHG that is glycosylated with arabinose is bold/italic/dotted underlined; the tri-arabinan is found on the double proline site within this sequence. Other prolines are bold/underlined, and double prolines are bold/double underlined. (b) MS and MS/MS analysis of pronase digestion products. LTB-MUC1-6His gel fractions were pooled and subjected to digestion with pronase. One small peptide with the amino acid sequence GSTAPPAHG comprising proline residues 113 and 114 was identified with three arabinose residues attached. This glycopeptide was only found in LTB-MUC1-6His samples but not in LTB-6His.

(a)

(b)
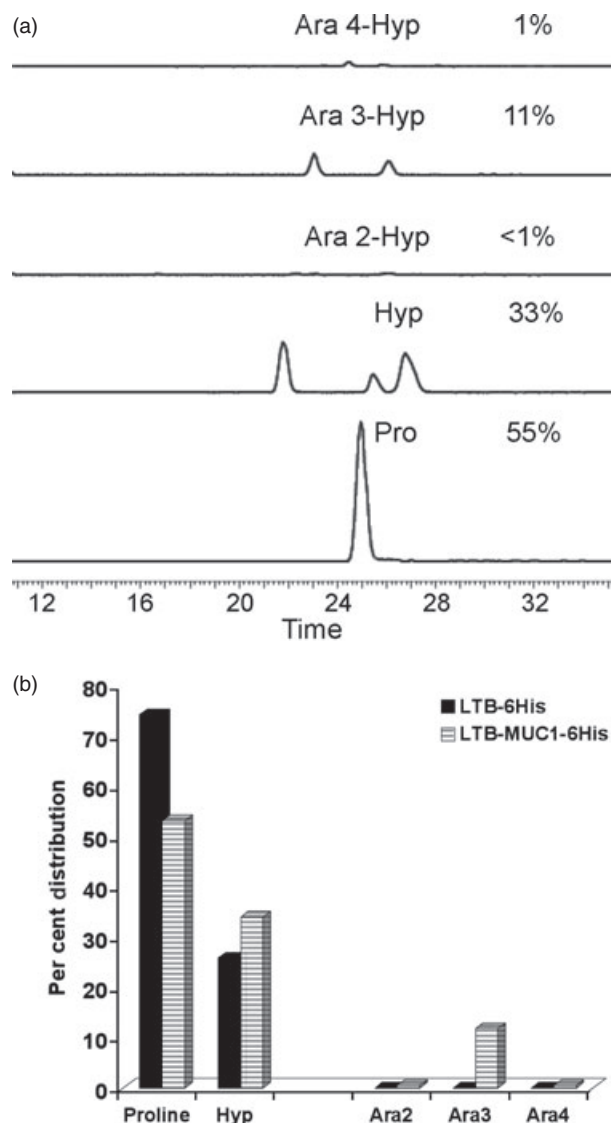
Figure 6 Analysis of Ara-Hyp species in LT-MUC1-6His. (a) Carbon-LC-ESI-MS analysis of LTB-MUC1-6His after alkaline degradation. A high percentage of the prolines as hydroxyprolines (33%) or glycosylated hydroxyprolines, mainly with a tri-arabinan (11% of total prolines) were observed. Hyp and glyco-Hyp give two peaks containing different conformers. (b) Quantitative comparison of the content of Proline (Pro), hydroxyproline (Hyp) and glycosylated hydroxyproline between LTB-6His and LTB-MUC1-6His. Both samples contained proline and hydroxyproline. LTB-MUC1-6His contained more hydroxyproline than LTB-6His and contained Ara-Hyp glycopeptides, mainly with three arabinose residues. Ara2, Ara3 and Ara4 designate a Hyp with 2, 3 and 4 Ara residues, respectively.

The transient magnICON™ agroinfiltrated *N. benthamiana* plants produced LTB-MUC1 at approximately 3% TSP. We observed signs of toxicity as a result of LTB expression, which has been previously reported in stable plant transformants–expressing LTB (Mason *et al.*, 1998). Overexpression of certain proteins in transgenic plants can interfere with normal plant processes and cause altered phenotype or cell death. This is more evident in the magnICON™ viral vector system because of extremely high expression levels. Although the magnICON™ system has the potential to produce a protein of interest up to

80% TSP (Marillonnet *et al.*, 2004), the actual amount produced depends on the specific protein and must be evaluated on a case by case basis.

Labile nontoxic B subunit Western blot revealed that magnICON™-expressed LTB-MUC1-6His contained the expected 16-kDa protein and two higher molecular weight proteins (18 and 20 kDa) that were not expected. These higher molecular weight proteins could not be ascribed to variations in mRNA processing, as Northern analysis (Figure 1b) showed only the expected size transcripts. However, the plant-derived LTB is targeted to the endoplasmic reticulum (ER) through its cleavable amino-terminal signal peptide, which targets it to the periplasmic space in *E. coli* (Mason *et al.*, 1998). Within a plant system, LTB-MUC1 is predicted to pass through the vesicular pathway from the ER to the Golgi apparatus and to undergo post-translational modifications.

Glycan analysis using RP-HPLC clearly showed that the high molecular weight bands are glycosylated forms of LTB-MUC1 (Figure 4). Specifically, pronase digestion revealed the glycans are tri-arabinans linked to hydroxyprolines within the MUC1 tandem repeat sequence GSTA**PP**AHG (Figure 5). These results are consistent with the Hyp contiguity hypothesis (Kieliszewski and Lamport, 1994), which defines general rules to predict Pro hydroxylation and Hyp O-glycosylation. The target motif for *O*-glycosylation consists of at least two [Ala∕Ser∕Thr∕Val]-Hyp repeats, with two consecutive Hyp not separated by more than 11 amino acids (Tan *et al.*, 2003). The Hyp contiguity hypothesis predicts arabinosylation of contiguous Hyp residues and galactosylation of clustered, noncontiguous Hyp residues. *O*-Hyp glycosylation occurs in two distinct modes, Hyp arabinosylation and Hyp galactosylation (Shpak *et al.*, 2001). Hyp arabinosylation results in short (usually 1–4 residues) neutral, linear oligosaccharides of ʟ-arabinofuranose (Hyp-arabinosides). Hyp galactosylation results in addition of much larger arabinogalactan heteropolysaccharides (Hyp-polysaccharides). Hyp-arabinosides have also been shown to enhance polyproline II helix content, which have been implicated to contribute to structural integrity and an increase in thermal stability of hydroxyproline-rich glycoproteins (HRGPs; Owens *et al.*, 2010). A recent study showed Hyp∕Pro conversion and O-linked arabinosylation on a recombinant therapeutic protein expressed in transgenic plants (Karnoup *et al.*, 2005). In conjunction with our study, these reports outline the significance of post-translational modification of recombinant proteins in plant systems and warrant more detailed characterizations in future studies and applications.

Glycosylation is known to alter essential biological functions, such as immunogenicity. The essential role glycosylation plays is clearly seen in the human TAA MUC1. In breast cancer, MUC1 has shorter and less complex chains (core 1 type) compared to the highly branched and extended chains (core 2 type) found in normal cells, resulting in the apparent unmasking of the peptide core and revealing immunodominant peptide sequences. A recent study showed that a tumour cell line exhibited a higher density of *O*-glycosylation, which was attached to 95% of available sites, whereas MUC1 from milk was glycosylated on 55% of the available sites (Hanisch and Muller, 2000). This study challenges earlier studies suggesting that underglycosylation of cancer cell MUC1 was because of both reduction in glycan chain length and reduced density. The observations from the recent study suggest that the altered glycosylation induces a conformational change in the peptide backbone, which
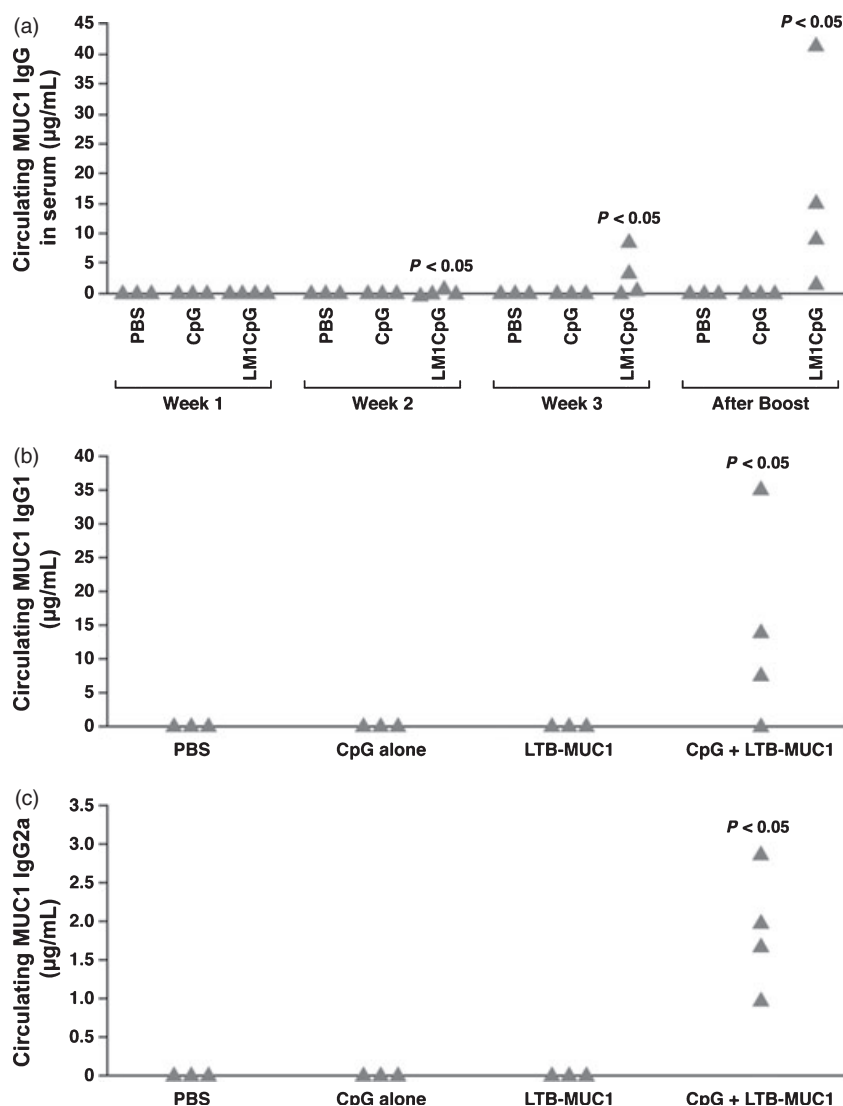
Figure 7 Parenteral immunization of MUC1.Tg mice with purified magnICON™ generated LTB-MUC1-6His. (a) Serum analysis for MUC1-specific IgG in MUC1.Tg mice treated with: phosphate buffer saline vehicle control (PBS); 100 μg CpG ODN (CpG); 100 μg CpG + LTB-MUC1-6His (LM1CpG). Serum samples were derived from blood drawn 1 week after each immunization (days 0, 7 and 14) and boost (day 35). (b) Serum MUC1-specific IgG1 and IgG2a (c) in MUC1.Tg mice treated with: PBS; 100 μg CpG ODN (CpG); LTB-MUC1-6His (LTB-MUC1); 100 μg CpG + LTB-MUC1-6His (CpG + LTB-MUC1). Anti-MUC1 titres (IgG, IgG1 and IgG2a) in mice treated with CpG + LTB-MUC1 are significantly higher ($P < 0.05$) than the PBS control group. Individual mouse data are shown (n = 3–5 mice per group).

reveals immunodominant epitopes. Other evidence points to the importance of tumour-associated MUC1 glycopeptides. The hypoglycosylated tumour-associated form of MUC1 contains abnormal mono- and disaccharide antigens, such as Tn (Gal-NAc-O-S/T) and T (Gal-GalNAc-O-S/T), which are not normally exposed on healthy cells or tissues, conversely 90% of carcinomas express these tumour-associated peptides (Springer, 1997). A recent study showed that incorporation of tumour-associated Tn antigens on MUC1 TR peptides induced glycopeptides-specific responses and boosted previously suppressed MUC1 peptide-specific T cell and antibody responses in the MUC1.Tg mice (Ryan *et al.*, 2009). It is of interest to note that Hyp arabinosylation is also made up of short oligoarabinosides, which may be similar to the shorter and less complex glycan chains found on the tumour form of MUC1. If this explanation holds true, we can then speculate that the glycosylation of LTB-MUC1-6His alters the peptide backbone in a similar fashion, thereby mimicking the tumour form of MUC1, and induces a robust response capable of breaking self-antigen tolerance.

Our study with the anti-MUC1 BC2 monoclonal antibody showed that the plant-expressed unglycosylated LTB-MUC1-6His

was not detected by BC2 (Figure 3c); thus, glycosylation is a likely requirement for BC2 binding. This observation suggests that in the unglycosylated form, the MUC1 epitope may undergo alternative folding that diminishes its antigenicity. The absence of signal at 16 kDa in the anti-His Western blot (Figure 3b) supports this idea, because the 6His was attached to the C-terminus of the MUC1 tandem repeat, and the 16-kDa species bound to the metal affinity resin. Therefore, the post-translation glycosylation of MUC1 might induce a conformational change in the polypeptide backbone that allows the MUC1 and His epitopes to be available for antibody detection.

We tested the immunogenicity of LTB-MUC1-6His in a human MUC1-tolerant mouse model. In the context of parenteral delivery, LTB serves as a carrier protein because small molecules with a molecular weight of <2–5 kDa are not usually immunogenic, even when administered in the presence of adjuvant. To generate an immune response to these compounds, it is necessary to attach them to a protein or other compound that is immunogenic, which in our case is LTB. Animal studies using the MUC1.Tg mouse model injected with the purified LTB-MUC1-6His with the adjuvant CpG ODN

showed anti-MUC1 IgG, IgG1 and IgG2a responses (Figure 7a–c, respectively).

To our knowledge, this is the first report to demonstrate expression of a breast cancer antigen in a plant system. After immunization of the MUC1-tolerant mouse with the plant LTB-MUC1-6His antigen, we observed abrogation of self-antigen tolerance. Breaking immune tolerance is a remarkable achievement in a cancer vaccine and illustrates that a plant-derived tumour-associated antigen is equivocal to the human antigen in the context of immune recognition. This study supports the utilization of plants as an alternative production system for therapeutic and vaccine formulations.

Experimental Procedures

LTB-MUC1 design and expression plasmid construction

The MUC1 TR antigen (MUC1—STAPPAHGVTSAPDTRPAPGST-APPA) contains the H-2D^b MHC class I–restricted immunodominant epitope (APGSTAPPA) and the H-2K^b restricted epitope (SAPDTRPAP). We modified the MUC1 coding sequence to contain codons preferred by dicotyledonous plant genes, eliminate aberrant mRNA processing and destabilization signals, and minimize secondary structure hairpins. To decrease the chance of steric hindrance in the fusion protein, the linker Gly-Pro-Gly-Pro (GPGP) was inserted between the LTB and MUC1 sequences. The GPGP-MUC1 coding sequence was constructed using four oligonucleotides, each approximately 50 bp long that, upon annealing and assembly of the nucleotides as described by Stemmer *et al.* (1995), contained a 5′ blunt end and a 3′ KpnI restriction site. The pLTB-L-plasmid (Walmsley *et al.*, 2003) containing the plant-optimized LTB sequence was prepared for insertion of the GPGP-MUC1 peptide by cutting and blunting the BbsI site, followed by digestion with KpnI. The LTB-MUC1 fusion gene is deposited in GenBank accession number DQ854816.

The magnICON^{TM} expression system is based on in planta assembly of functional viral vectors from separate pro-vector modules previously described by Marillonnet *et al.* (2004). The integrase module pICH14011 and the 5′ module pICH15879 are shown in Figure 1a. The 3′ module pICH-LTB contains the LTB gene inserted into the multiple cloning site of the pICH11599 module (Marillonnet *et al.*, 2004). The LTB-MUC1 gene from pJPLM1 was cut with KpnI/BglII, blunted at the KpnI site with T4 DNA polymerase and inserted into pICH-LTB cut with BamHI/BglII and blunted at the BamHI site, generating pICH-LM1 (Figure 1a). The His-tagged LTB-MUC1 fusion gene was generated by PCR using pICH-LM1 as the template and the reverse primer MHisRev (5′-AAGT**CTGCAG**TTA*ATGGTGATGGTG ATGATGGTG*TGCAGGTGGTGCAGTAG-3′), which contains segments of the C-terminus of the MUC1 gene (underlined) along with 6His sequence (italicized) and a PstI restriction site (bold) and the forward primer nosR (5′-TGCGGTTCTGTCAGTTCCAAA-3′). The resulting PCR fragment and the vector pICH-LTB were cut with NcoI/PstI and ligated to generate pICH-LM1His (Figure 1b). The His-tagged LTB gene was constructed by PCR using pICH-LM1 as the template and the reverse primer LTB6H-Sac (5′-GGGAGCTCTTAATGGTGATGGTGATGGTGACTAGGTCCGGA-GTTCTCCATGCTGATGGC), which introduces a 3′ SacI site, a 6His sequence and the linker 'SGPS', and the forward primer nosR. The resulting PCR product and pICH11599 were digested with NcoI and SacI and ligated to make pICH-LTBHis.

Agroinfiltration procedure for magnICON^{TM} viral vector transformation

The magnICON^{TM} pro-vector modules containing the LTB-MUC1 gene (Figure 1a) were prepared from cultures of *E. coli* DH5α and electroporated into *Agrobacterium tumefaciens* strain GV3101. The *Agrobacterium* containing the pro-vector modules were grown in YENB medium (7.5 g⁄L yeast extract, 8 g⁄L nutrient broth) supplemented with rifampicin (60 mg⁄L) and carbenicillin (50 mg⁄L) at 28 °C. Overnight cultures (OD_{600} = 0.5–1.0) were adjusted to a final OD_{600} of 0.1, and equal volumes of the pro-vector modules (integrase, 5′ module, 3′ module 1 : 1 : 1) were mixed, sedimented (5 min, 5000 **g**) and resuspended in infiltration buffer (10 mM MES, pH 5.5 and 10 mM $MgSO_4$) at 0.1 OD_{600}. Leaves of greenhouse-grown *N. benthamiana* were infiltrated using a syringe without a needle as described previously (Huang *et al.*, 2006).

Nucleic acid analysis

Total RNA samples were isolated from 500 mg of leaf tissue using RNAqueous (Ambion, Austin, TX) and Plant RNA isolation Aid (Ambion, Austin, TX). The RNA samples were quantified spectrophotometrically. Ten micrograms of total RNA was denatured with formaldehyde⁄formamide, electrophoresed on a 1% agarose MOPS-acetate-EDTA gel, capillary transferred to a Zeta probe membrane (Bio-Rad Laboratories, Hercules, CA) as described by Sambrook and Maniatis (1981) and fixed by UV cross-linkage. The membrane was then washed in 5% acetic acid, stained with 0.25% methylene blue in 0.3 M sodium acetate (pH 5.2) and destained with RNase-free water. Hybridization and membrane detection were performed as per the manufacturer's instructions using an LTB-MUC1-specific probe (DIG wash and block buffer set and DIG Luminescent Detection Kit; Roche Applied Sciences, Indianapolis, IN) using a probe concentration of 5 μL⁄mL. Labelled membranes were visualized after exposing to KODAK BioMax MS film.

Metal affinity purification of His-tagged LTB-MUC1

His-tagged LTB-MUC1 fusion protein was purified using TALON Metal Affinity Resin (BD Clontech, Mountain View, CA) at 4 °C under gravity. Ten grams of fresh leaf material was snap-frozen with liquid N_2 and homogenized in a mortar and pestle with 40 mL lysis buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 0.5% CHAPS) and one tablet of Complete, Mini, EDTA-Free Protease Inhibitor Cocktail (Roche Applied Sciences, Indianapolis, IN), transferred to 50-mL Falcon tubes and kept on ice for 10 min. After 15-min centrifugation (5000 **g**), supernatant was filtered with Miracloth (Calbiochem, San Diego, CA) and then with 0.45-μm filters. Filtered supernatant was added to TALON resin (2 mL bed volume) equilibrated previously with lysis buffer in a 25-mL chromatography tube. The flow through was passed through the column twice before discarding. Columns were washed with ten volumes of wash buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 5 mM imidazole, 0.5% CHAPS) and then eluted with six volumes of elution buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 150 mM imidazole, 0.5% CHAPS). Eluted fractions were desalted and then concentrated with the Pierce iCON Protein Concentrator 9K MWCO (Thermo Fisher Scientific, Rockford, IL).

Galactose affinity chromatography

Leaf material was homogenized in a blender with ice-cold extraction buffer [20 mM sodium phosphate buffer (pH 7.4),

150 mM NaCl, 0.1 M sodium ascorbate, SigmaFast™ Protease Inhibitor Cocktail Tablet (Sigma-Aldrich, St. Louis, MO), 0.1% Triton]. Used 500 mL extraction buffer per 100 g leaf material. The plant material was then spun on ice for 30 min, to allow full homogenization. The material was then filtered through cheese cloth and centrifuged at 8000 ***g*** for 20 min. Transferred the supernatant into a new tube (trying not to disturb the pellet) and spun again for 20 min at 8000 ***g***. Used Immobilized D-Galactose (Thermo Fisher Scientific, Rockford, IL) for galactose column and equilibrated with buffer (20 mM sodium phosphate buffer (pH 7.4), 150 mM NaCl). The plant supernatant was then run through the column at 4 °C, and the flow through was collected. The column was washed ten times the bed volume with buffer [20 mM sodium phosphate buffer (pH 7.4), 150 mM NaCl] and eluted with five volumes of elution buffer (600 mM galactose, 20 mM sodium phosphate buffer (pH 7.4), 150 mM NaCl). The eluted fractions were then further purified using the Talon metal affinity column as described above.

Acetone precipitation

The purified LTB-6His and LTB-MUC1-6His were precipitated with acetone prior to shipment and further glycan analysis. To precipitate the protein samples, we added five times the sample volume of cold acetone. The tube was vortexed and then incubated for 1 h at −20 °C. The samples were then centrifuged for 10 min at max speed (14 000 ***g***). The supernatant was disposed of, and the pellet was allowed to air dry at room temperature for 30 min.

Ganglioside capture ELISA

Ganglioside-dependent ELISA was performed according to Haq *et al.* (1995). Volumes of 50 μL in microtiter plates (Costar 3590; Fisher Scientific, PA) were used throughout. Samples were assayed in two replicates of varying dilutions in PBSTM (1% dry milk + PBS + 0.05% Tween-20) of each extract. Plates were washed three times with PBST (PBS + 0.05% Tween-20) and incubated with either 1 : 1000 rabbit anti-LTB serum (Benchmark Biolabs, Overland Park, KS) or mouse anti-MUC1 monoclonal antibody BC2 (Xing *et al.*, 1989) in PBSTM for 1 h at 37 °C. Wells were incubated with goat anti-rabbit IgG–horseradish peroxidase (HRP) conjugate (Sigma, St Louis, MO) diluted 1 : 12 000 in PBSTM for 1 h at 37 °C and developed with TMB Peroxidase EIA Substrate kit (Bio Rad, Hercules, CA) for 5 min at 23 °C. Absorbance at 450 nm was measured, and ELISA data were converted to micrograms LTB per gram of fresh weight by reference to a standard curve constructed using purified bacterial LTB (provided by Benchmark Biolabs). Although ganglioside-dependent ELISA was used for detecting MUC1 in the plant samples, the MUC1 standards were directly bound to the plates. The standard for MUC1 detection was a synthetic MUC1 tandem repeat peptide (cys-STAPPAHGVTSAPDTRPAPGS-TAPPA) synthesized at Arizona State University, Department of Biochemistry, Tempe, AZ.

Western blot to detect LTB-MUC1

Western analysis to detect LTB and MUC1 from plant protein extract was performed as described by Walmsley *et al.* (2003). The membrane was blocked with 3% PBSTM (3% dry milk + PBS + 0.1% Tween-20) overnight, and LTB was detected with rabbit anti-LTB serum (1 : 5000 dilution in 1% PBSTM), followed by an anti-rabbit IgG–HRP (1 : 9000 in 1% PBSTM;

Sigma). To detect MUC1, we used the MUC1-specific antibody BC2 (Xing *et al.*, 1989) (1 : 5000 dilution in 1% PBSTM), followed by an anti-mouse IgG–HRP conjugate (1 : 8000 dilution in 1% PBSTM; Pierce Biotechnology, Rockford, IL). To detect the 6His-tag, we used nickel-conjugated HRP (Pierce/Thermo Scientific HisProbe-HRP, product #15165, 1 : 1000 dilution in 1% PBSTM). Detection was performed using the Amersham ECL + kit (Bio-Rad Laboratories) as per manufacturer's instructions.

In silico analyses

To determine whether our LTB-MUC1 construct has potential *N*-glycosylation sites, we used the NetNGlyc 1.0 server, which predicts *N*-glycosylation sites in human proteins using artificial neural networks that examine the sequence context of Asn-Xaa-Ser/Thr sequons (http://www.cbs.dtu.dk/services/NetNGlyc/). To determine whether our LTB-MUC1 has potential O-glycosyation sites on Ser/Thr residues, we used NetOGlyc 3.1 server, which produces neural network predictions of mucin type GalNAc-O-glycosylation sites in mammalian proteins (http://www.cbs.dtu.dk/services/NetOGlyc/).

Monosaccharide analysis

Samples were purified by galactose affinity followed by metal affinity chromatography, and then subjected to SDS–PAGE, Coomassie-stained bands were excised, dried, hydrolysed for 3 h with 2 M TFA and the released monosaccharides were derivatized with anthranilic acid. The labelled sugars were analysed by reverse-phase high-performance liquid chromatography (RP-HPLC) with fluorescence detection (Anumula KR, 1994 Anal. Biochem.). Blank gel pieces were used as background controls.

Amino acid analysis for the presence of Hyp and glycosylated Hyp

Samples were subjected to SDS–PAGE, and the stained bands were then excised. Excised bands were pooled and then subjected digestion with pronase, which has a very low specificity and hence generates small, overlapping peptides. This was followed by analysis using MS and MS/MS. For alkaline degradation, the samples were pooled and then dissolved and subjected to degradation with Ba(OH)$_2$. The resulting amino acids and glyco-amino acids were dansylated and analyzed by carbon-LC-ESI-MS (Leonard *et al.*, 2010).

Parenteral immunization with purified magnICON™-expressed LTB-MUC1-6His

MUC1.Tg mice (6–8 weeks old) were bred in-house (Rowse *et al.*, 1998) at the Mayo Clinic Scottsdale Natalie Schafer Transgenic Animal Facility in pathogen-free conditions. Groups of mice (*n* = 5) were injected intraperitoneally (i.p.) with 2–20 μg of LTB-MUC1-6His, or 2–20 μg LTB-MUC1-6His + 100 μg CpG ODN (1826; Coley Pharmaceutical Group, Wellesley, MA). Control groups were injected i.p. with PBS (pH 7.4), or 100 μg CpG ODN or control plant extract purified with TALON Resin. Mice were immunized on days 0, 7, 14 and 35. Tail blood from individual mice was collected on a weekly basis until termination (day 70).

Determination of IgG, IgG1 and IgG2a titres in serum

ELISA plates (Falcon #353911; BD Biosciences Discovery Labware, Bedford, MA) were coated with 5 μg/50 μL/well of the

chemically synthesized MUC1 peptide in PBS and incubated overnight at 37 °C. After three washes with PBST, plates were blocked with 10% foetal calf serum (FCS) in PBS for 1 h at 37 °C. Serum samples were serially diluted in the plates using 10% FCS/PBS, starting at 1 : 20. The plates were incubated for 1 h at 37 °C before washing and incubation with goat anti-mouse IgG–HRP (Pierce Biotechnology), diluted 1 : 20 000 in 10% FCS/PBS, for 1 h at 37 °C. For IgG1 and IgG2a, the plates were incubated with HRP-conjugated goat anti-mouse IgG1 or anti-mouse IgG2a antibodies (Southern Biotech, Birmingham, AL), diluted 1 : 2000 in 10% FCS/PBS, for 1 h at 37 °C. Plates were developed with TMB peroxidase substrate (Bio-Rad Laboratories) for 5 min. The reaction was stopped with 1 N H_2SO_4, and the absorbance read at 450 nm. Concentrations of serum anti-MUC1 were determined by linear regression from a standard curve of BC2, a mouse monoclonal antibody with epitopes in the tandem repeat (TR) domain on MUC1 (Xing *et al.*, 1989). The anti-LTB antibody ELISA was performed as described for the anti-MUC1 ELISA except plates were coated with ganglioside (Sigma-Aldrich G3018) 0.5 μg/50 μL per well and incubated overnight at 23 °C, before adding 125 ng of LTB in 50 μL per well (Benchmark Biolabs). Concentrations of serum anti-LTB were determined by linear regression from a standard curve of rabbit anti-LTB, which was purified using the HiTrap Protein G HP affinity column (Amersham Biosciences, Piscataway, NJ), and then quantified for protein concentration using absorbance at 280 nm. Data are presented as units/ml, where 1 unit is defined as 1 ng/mL rabbit anti-LTB IgG equivalent.

Statistical analysis

For the parenteral immunization study, Fisher's exact test was applied to determine the incidence of MUC1-specific antibody concentrations between the PBS and CpG ODN control groups to the LTB-MUC1 and LTB-MUC1 + CpG ODN treatment groups. Results were considered statistically significant if $P < 0.05$.

Acknowledgements

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