Linear and Branched Glyco-Lipopeptide Vaccines Follow Distinct Cross-Presentation Pathways and Generate Different Magnitudes of Antitumor Immunity

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Abstract

Background: Glyco-lipopeptides, a form of lipid-tailed glyco-peptide, are currently under intense investigation as B- and T-cell based vaccine immunotherapy for many cancers. However, the cellular and molecular mechanisms of glyco-lipopeptides (GLPs) immunogenicity and the position of the lipid moiety on immunogenicity and protective efficacy of GLPs remain to be determined.

Methods/Principal Findings: We have constructed two structural analogues of HER-2 glyco-lipopeptide (HER-GLP) by synthesizing a chimeric peptide made of one universal CD4+ epitope (PADRE) and one HER-2 CD8+ T-cell epitope (HERε20-429). The C-terminal end of the resulting CD4+CD8 chimeric peptide was coupled to a tumor carbohydrate B-cell epitope, based on a regionselectively addressable functionalized templates (RAFT), made of four α-GalNAc molecules. The resulting HER glyco-peptide (HER-GP) was then linked to a palmitic acid moiety, attached either at the N-terminal end (linear HER-GLP-1) or in the middle between the CD4+ and CD8+ T-cell epitopes (branched HER-GLP-2). We have investigated the uptake, processing and cross-presentation pathways of the two HER-GLP vaccine constructs, and assessed whether the position of linkage of the lipid moiety would affect the B- and T-cell immunogenicity and protective efficacy. Immunization of mice revealed that the linear HER-GLP-1 induced a stronger and longer lasting HERε20-429-specific IFN-γ producing CD8+ T cell response, while the branched HER-GLP-2 induced a stronger tumor-specific IgG response. The linear HER-GLP-1 was taken up easily by dendritic cells (DCs), induced stronger DCs maturation and produced a potent TLR-2-dependent T-cell activation. The linear and branched HER-GLP molecules appeared to follow two different cross-presentation pathways. While regression of established tumors was induced by both linear HER-GLP-1 and branched HER-GLP-2, the inhibition of tumor growth was significantly higher in HER-GLP-1 immunized mice (p<0.005).

Significance: These findings have important implications for the development of effective GLP based immunotherapeutic strategies against cancers.

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Introduction

Aberrant glycosylation leads to the expression of abnormal tumor associated carbohydrate antigens (TACAs) and are considered as a unique target for tumor-specific IgG/IgM antibodies [1,2,3,4,5]. The most common TACA derived B-cell epitope is Tn antigen [a precursor of Thomsen-Friedenreich or TF antigen], also known as GalNAc [5,6]. Tn-specific IgG/IgM are detected in up to 90% of human carcinomas, but their level and affinity is weak [3]. When administered alone, TACAs activate the antibody secreting B cells weakly [3,7]. Similarly, tumor-specific CD8+ T cells are also detected in cancer patients, but their level and function are not sufficient enough to control tumor progression [6]. Therefore, immunotherapeutic vaccines that can boost the induction of tumor-specific CD8+ T cells and boost their function are required for tumor protection. An ideal immunotherapeutic cancer vaccine should comprise both TACA derived carbohydrate B-cell epitope and tumor associated antigen (TAA) derived CD8+ T-cell epitopes to boost the sub-optimal antitumor B- and T cell immune responses often detected in cancer patients [8,9,10,11].

Molecularly defined and human compatible self-adjuvanting vaccines that are capable of inducing tumor-specific antibody and CD8+ T-cell immunity are limited [reviewed in [1] and [12]]. The low molecular weight lipid molecule (palmitic acid) is derived from a
immunologically active lipoprotein of *Escherichia coli* origin and has been widely used, as an adjuvant, to enhance the immunogenicity of both peptide T-cell epitopes and carbohydrate B-cell epitopes. Palmitic acid (PAM) also acts as a biological ligand for toll receptor 2 (TLR-2) that is expressed on the surface of antigen presenting cells, such as dendritic cells, and enhances their phenotypic and functional maturation. Dendritic cells cross-present exogenous palmitic acid-tailed peptide epitopes (i.e. lipopeptides), associate them with their MHC class I molecules, and present them to prime CD8+ T cells. The resulting PAM-HER 420–429-RAFT is designated as "HER-GP".

The corresponding non-lipidated structural analog of HER-GP were assembled from compound 1 using convergent ligation chemistry based on oxime and disulfide bond formation described earlier. The cyclopeptide RAFT scaffold 1 displays the cluster of Tn antigen to ensure efficient antigen delivery andcontains a cystein-Npsys moiety on the other addressable domain. This activated cysteine residue permits the conjugation of peptide or lipopeptide-containing cysteine at the C-terminal end. The final disulfide coupling reaction between compound 1 (Fig. 1A) and the peptide HER420-429-PADRE 2 or lipopeptides PAM-HER420-429-PADRE 3 and HER420-429-PADRE 4 was performed under argon gas in a mixture of isopropanol and sodium acetate buffer. The purified HER-GP glyco-peptide, the linear HER-GLP-1; and the branched HER-GLP-2 glyco-lipopeptides were homogeneous in solution and their expected primary molecular weights were derived by electrospray mass spectrometry (Fig. 1B). Multiple freeze-thaw cycles, over a period of one year, did not disrupt the physicochemical properties of the HER-GLP vaccine in solution.

Finally, the linear HER-GLP-1, the branched HER-GLP-2 and their parent non-lipidated HER-GP were labeled unequivocally by fluorescence probe Alexa Fluor 488 to study their entry into immature dendritic cells by confocal microscopy.

**Branching HER-GLP-2 induced stronger RAFT-specific IgGs than linear HER-GLP-1**

To compare the B-cell immunogenicity of linear HER-GLP-1 and branched HER-GLP-2 molecules (Fig 2A), B10.D1 mice (10 mice/group) were immunized subcutaneously with equimolar amount of either linear HER-GLP-1 (Group 1) or branched HER-GLP-2 (Group 2) vaccine constructs in adjuvant-free saline. A third group of ten B10.D1 mice was immunized with the non-lipidated HER-GP analog in adjuvant-free saline (Group 3) and used as control. A fourth group of ten B10.D1 mice was injected subcutaneously with saline alone (Group 4, Mock). No adverse reaction such as local inflammation at the sites of injection or weight loss was observed in any immunized mice rendering the safety of these adjuvant-free vaccine formulations. Ten days after the immunization, the serum IgG levels specific to carbohydrate RAFT were determined in each group by ELISA. Significant levels of RAFT-specific IgG were induced in both linear HER-GLP-1 and branched HER-GLP-2 immunized mice (Fig. 2B) p<0.05 and p<0.01, respectively. However, the branched HER-GLP-2 appeared as a better immunogen than the linear HER-GLP-1, suggesting that the position of the lipid moiety affects the magnitude of antibody responses generated by the GLP vaccines. Interestingly, immunization of mice with non-lipidated HER-GP did not induce any significant level of RAFT-specific IgG responses (p>0.05), revealing the requirement for the built-in palmitic acid in generating carbohydrate specific IgG Abs.

Constructs missing any of the four components did not produce Abs response suggesting that it is crucial to have all the components linked within one molecule (not shown).

The IgGs induced by both linear HER-GLP-1 and branched HER-GLP-2 construct bind human breast tumor cell line MCF7 expressing Tn molecules (Fig. 2C and 2D). However, higher (350 fold) binding was observed for branched HER-GLP-2 induced IgGs than linear HER-GLP-1 induced IgGs (150 fold) when compared to non-immune control IgGs (p<0.01). Under identical experimental conditions, IgGs induced by both HER-GLP-1 and HER-GLP-2 immunogen did not show any binding to T2 and RS cell lines that do not express Tn molecules (not shown).

Taken together, these results indicate that while both linear HER-GLP-1 and branched HER-GLP-2 molecules induced RAFT-specific IgGs that bind to human tumor cell lines expressing the native Tn antigen, the branched HER-GLP-2 appeared to be a stronger B-cell immunogen than the linear HER-GLP-1.


Linear HER-GLP-1 induced stronger and long lasting HER420-429-specific IFN-γ-producing CD8+ T cell responses than branched HER-GLP-2

Groups of B10.D1 mice (10/group) were immunized subcutaneously three times at fourteen-day intervals with equimolar amount of linear HER-GLP-1 or branched HER-GLP-2 (Fig 3A). As a control a third group of ten B10.D1 mice was immunized with the non-lipidated HER-GP analog. A fourth group of ten B10.D1 mice was injected subcutaneously with saline alone (Mock). Ten days after the third immunization, HER420-429-specific CD8+ T cells were evaluated in the spleen.

Spleen-derived cells were re-stimulated in vitro with HER420-429 peptide for four days and HER420-429-specific IFN-γ producing CD8+ T cell responses were measured by ELISPot assays. As shown in Fig 3B, both linear HER-GLP-1 and branched HER-GLP-2 immunized mice developed significant number of HER420-429 specific IFN-γ producing CD8+ T cells when compared with mock-immunized control mice ($p<0.05$). However, the linear HER-GLP-1 showed higher number of HER420-429 specific IFN-γ producing CD8+ T cells than the branched HER-GLP-2, suggesting that the position of the lipid moiety in the GLP construct does affect the magnitude of CD8+ T cell responses. As expected, the HER-GP immunized mice showed a non-significant number of HER420-429 specific CD8+ T cells when compared to mock-immunized control mice ($p>0.05$), further underlining the requirement for the built-in palmitic acid immuno-adjuvant for the induction of the T-cell responses. Constructs missing one of the four components or non-covalent mixtures of the parts did not induce a T cell response suggesting that it is crucial to have all the components linked within one molecule (not shown).

We next performed a kinetic measurement of HER420-429 specific IFN-γ-producing CD8+ T cells in mice immunized with both linear HER-GLP-1 and branched HER-GLP-2 for up to 60 days post immunization. We observed that the percentage of HER420-429-specific IFN-γ-producing CD8+ T cells was gradually increased and reached the peak 4.3% for HER-GLP-1 and 3.5% for HER-GLP-2 on day 28 (Fig. 3C). Thereafter, the percentage of HER420-429-specific IFN-γ-producing CD8+ T cells were gradually decreased but instead of completely declining, a certain percentage (2.5% for HER-GLP-1 and 1.8% for HER-GLP-2) of HER420-429-specific IFN-γ-producing CD8+ T cells were maintained up to day 60. Taken together, these results indicate that while both linear HER-GLP-1 and branched HER-GLP-2 molecules are capable of inducing a long-lasting HER420-429 specific CD8+ T-cell response, the linear HER-GLP-1 appeared to be a stronger T-cell immunogen than the branched HER-GLP-2. These results illustrate the importance of the position of palmitic acid moiety on HER-GLP vaccine construct in terms of maintaining the long lasting IFN-γ-producing CD8+ T cells. Unlike the CD8+ T cell responses, PADRE-specific CD4+ T cell proliferative responses were not affected by the position of the lipid moiety (not shown).

Regression of established tumors following immunotherapeutic immunization with linear HER-GLP-1 and branched HER-GLP-2 vaccines

The immunotherapeutic efficacy of self-adjuvanting linear HER-GLP-1 and branched HER-GLP-2 vaccine molecules were compared by assessing tumor growth and mice survival rate. To develop tumor, female B10.D1 mice (10 per group) were implanted subcutaneously in the mammary fat pad with $1 \times 10^5$ NT2 cells. Eight to ten days later, when tumor diameter reached 3–4 mm, mice were immunized subcutaneously four times at seven day intervals with the linear HER-GLP-1 (group 1), the branched HER-GLP-2 (group 2), both HER-GLP-1 and HER-GLP-2 (group 3) or injected with PBS alone (group 4, mock). As shown in Fig 4A, the tumor diameter (mm), which reflects the tumor progression, was significantly delayed in mice vaccinated with the linear HER-GLP-1 molecules compared with mock-immunized mice ($p<0.005$). However, therapeutic immunization with HER-GLP-2 did not lead to a significant reduction the tumor progression ($p>0.005$). The inhibition of tumor growth was significantly higher in linear HER-GLP-1 compared to branch HER-GLP-2 immunized mice ($p<0.005$). Interestingly, the protective efficacy detected in mice immunized with both HER-GLP-1 and HER-GLP-2 (group 3) was slightly higher than mice immunized with individual HER-GLP-1 and HER-GLP-2 constructs. The strong immunotherapeutic effect of the linear HER-GLP-1 was also evident from the survival graph (Fig. 4B). Of 10 mice vaccinated with the linear HER-GLP-1 and the branched HER-GLP-2 molecules, 8 of 10 and 6 of 10 were still alive after 8-week after tumor inoculation, respectively. Therapeutic immunization with both HER-GLP-1 and HER-GLP-2 protected 10 of 10 mice from death as compared to 0 of 10 mock-immunized mice ($p<0.005$).

Cytoplasmic uptake of linear HER-GLP-1 and branched HER-GLP-2 constructs by dendritic cells

In an effort to elucidate the mechanisms underlying the immunogenicity of linear HER-GLP-1 and branched HER-GLP-2 molecules, we determined the kinetics of their uptake by immature dendritic cells (DCs). Mouse bone marrow derived immature DCs were incubated with equimolar amount of Alexa Fluor 488 labeled HER-GLP-1 or HER-GLP-2 or non-lipidated HER-GP constructs. The uptake of each vaccine construct on DCs surface was analyzed by FACS and their cytoplasmic accumulation was visualized by confocal microscopy. Both linear HER-GLP-1 and branched HER-GLP-2 were efficiently taken up by DCs at a concentration as low as 1 μM (Fig. 3A left panel). Cytoplasmic accumulation of both linear HER-GLP-1 and branched HER-GLP-2, but not HER-GP, was visualized within 10 min of incubation (Fig. 3A, right panel). The Alexa Fluor 488-labeled HER-GP was unable to cross DC membrane even after several trials at higher concentration. This suggests that the attachment of a palmitic acid moiety play an important role in the entry of HER-GLP constructs into the cytoplasm of DCs.
Endocytosis assay uptake showed a total active uptake of HER-GP and HER-GLPs was measured by FACS and expressed by the difference in geometric mean of delta fluorescence intensity (ΔMFI) that resulted from subtracting the values obtained at 4°C from the values obtained at 37°C. Kinetic studies showed that the linear HER-GLP-1 was quickly taken up at 37°C by DCs within 10 minutes, whereas the uptake of branched HER-GLP-2 was relatively slower (Fig. 5B). Upon longer incubation at 37°C, the mean ΔMFI of the linear HER-GLP-1 construct on DCs was significantly higher than the branched HER-GLP-2 (p<0.05). After 30 min incubation, up to 85% of DCs were associated with the linear HER-GLP-1 and about 75% of DCs were associated with the branched HER-GLP-2 analog (Fig. 5A). A gradual increase in the ΔMFI of both constructs associated with DC was observed over time, reaching a plateau by 90 min (Fig. 5B). However, no further changes in the ΔMFI were observed between 90 to 120 min of incubation. The cytoplasmic entry of both linear HER-GLP-1 and branched HER-GLP-2 occurred at 37°C but
was inhibited at 4°C (not shown), indicating an active intracellular delivery mechanism. Together, these results show that both the linear HER-GLP-1 and branched HER-GLP-2, but not the non-lipidated HER-GP analog, were taken up quickly by the DCs and accumulated into DCs cytoplasm. The uptake of linear HER-GLP-1 appeared to be relatively faster compared to the branched HER-GLP-2 analog, suggesting that the position of the lipid moiety might affect the uptake of GLP constructs by DCs.

The linear HER-GLP-1 construct induced stronger dendritic cell maturation and followed by toll-like receptor 2-dependent T-cell activation

Next we sought to determine whether HER-GP and HER-GLP constructs are capable of inducing DC maturation and whether the position of the lipid moiety affects such maturation. Immature DCs were derived from mouse bone marrow and left untreated (none) or incubated in vitro for 48 hrs with an equimolar amount of either linear HER-GLP-1 or branched HER-GLP-2, or non-lipidated HER-GP and monitored the expression of cell surface major histocompatibility complex (MHC) class II, and B7 (CD80 and CD86) co-stimulatory molecules which are the well known phenotypic markers for DC maturation. Incubation of immature DCs with either linear HER-GLP-1 or branched HER-GLP-2 was associated with an increase in the production of IL-12p35 and TNF-α cytokines (Fig. 6B; P<0.005) in a concentration dependent manner. Under similar conditions,

Figure 3. Immunization with linear HER-GLP-1 induces stronger and long-lasting HER420-429-specific IFN-γ producing CD8+ T cell responses than branched HER-GLP-2. Three groups of B10.D1 mice (n = 10) were immunized subcutaneously three times with HER-GLP-1 (50 µM/mouse), HER-GLP-2 (50 µM/mouse) or HER-GP (50 µM/mouse) as shown in panel A) or injected with PBS alone (Mock) with an interval of 14 days. Ten days after the 3rd immunization, spleen cells were isolated and stimulated in vitro for 4 days with HER420-429 peptide and assayed for IFN-γ producing CD8+ T cells by ELISPOT. Mean values (± SD) of IFN-γ spot-forming CD8+ T cells were plotted against each group of mice and are shown in (B). Kinetics of HER420-429-specific IFN-γ producing CD8+ T cells were measured in mice immunized with HER-GLP-1, HER-GLP-2 and HER-GP from 0 to 60 days of post immunization and is shown in (C). The results are representative of three experiments.

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immature DCs incubated with the non- lipidated HER-GP construct failed to up-regulate the DC markers of maturation and did not produce the pro-inflammatory cytokines. These data suggest that the covalent linkage of the lipid moiety not only facilitated the uptake of the vaccine constructs but also supported phenotypic maturation of DCs.

In a separate ELISpot assay, we measured the number of HER420–429-specific IFN-\(\gamma\) producing CD8\(^+\) T-cells in the presence of bone marrow derived autologous immature DCs pre-pulsed with equimolar amount of linear HER-GLP-1 or branched HER-GLP-2 or non-lipidated HER-GP. Higher numbers of IFN-\(\gamma\)-secreting HER420–429 specific CD8\(^+\) T-cells were detected after stimulation with linear HER-GLP-1-pulsed DCs compared to stimulation with the branched HER-GLP-2-pulsed DCs (\(p<0.05\)) (Fig. 6C). None of the HER420–429 specific CD8\(^+\) T-cells were activated following incubation with immature DCs alone (none) or with DCs pulsed with a control irrelevant HSV-1 gB495–505 peptide (not shown).

To determine whether toll receptors (TLR-2 and TLR-4) on DCs is playing a potential role in stimulating HER420–429 specific CD8\(^+\) T-cells by linear HER-GLP-1 and branched HER-GLP-2 constructs, blocking experiments were performed. We incubated autologous immature DCs with either anti-TLR-2 or anti-TLR-4 mAbs, for 30 min before pulsing with the equimolar amounts of either linear HER-GLP-1, branched HER-GLP-2 or non-lipidated HER-GP. The anti-TLR-2 mAbs, but not the anti-TLR-4 mAbs, significantly abrogated the production of the IFN-\(\gamma\) by HER420–429 specific CD8\(^+\) T-cells (\(p = 0.002\)) indicating that both linear HER-GLP-1 and branched HER-GLP-2 activate DCs via a TLR-2 dependent pathway (Fig. 6C). Collectively, these results show that the phenotypic maturation of DCs induced by the linear HER-GLP-1 and branched HER-GLP-2 occurred through the TLR-2 signaling pathway.

The position of the lipid moiety profoundly affects the cross-presentation pathway of glyco-lipopeptides

To determine the cross-presentation pathways of HER-GLP-loaded DCs, we used specific antigen-processing inhibitors: brefeldin A, epoxomycin and monensin. Brefeldin A inhibits passage from the endoplasmic reticulum to the Golgi, the exocytic pathway [30] or inhibits the level of MHC class I molecule recycling [31]. Epoxomycin acts as a specific proteasome inhibitor [32] and inhibits the chymotrypsin-like activity and to a lesser extent the trypsin-like and peptidyl-glutaminyl peptide-hydrolyzing activities of the proteasome. Epoxomycin is very specific for the proteasome and does not inhibit non-proteosomal proteases such as trypsin, chymotrypsin, papain, cathepsin B, calpain, or tripeptidyl peptidase II [33]. The internalization of exogenous antigen by endocytosis and subsequent processing by DCs may occur through the endosomal pathway [27]. Monensin inhibits endosomal acidification, enzymatic degradation in the lysosomal compartments and as such might disturb endocytosis [27,34].

To assess the cross-presentation pathway, dendritic cells were first treated with brefeldin A, Epoxomycin or Monensin, as described in Materials and Methods, followed by the addition of linear HER-GLP-1, branched HER-GLP-2 or parent non-lipidated HER-GP construct. After overnight incubation, DCs were washed and added to the HER420–429 specific CD8\(^+\) T cells for additional 5 hrs. As a positive control, DCs were left untreated with antigen-processing inhibitors but were pulsed with linear HER-GLP-1, branched HER-GLP-2 or parent non-lipidated HER-GP constructs. HER420–429 specific CD8\(^+\) T cell responses were detected by ELISpot, as above. As shown in Fig. 7A, brefeldin A significantly inhibited the magnitude of IFN-\(\gamma\)-producing HER420–429 specific CD8\(^+\) T cells induced by both the linear HER-GLP-1 and branched HER-GLP-2 constructs. This indicates that the antigen processing of both the linear HER-GLP-1 and branched HER-GLP-2 constructs.
GLP-1 and branched HER-GLP-2 is governed by the passage from the endoplasmic reticulum to the Golgi. As shown in Fig. 7B, epoxomycin almost completely blocked the presentation of the linear HER-GLP-1 to HER420–429-specific CD8+ T cells. This indicated that the HER-GLP-1 is processed by the proteasome, which led to effective presentation of the HER420–429 epitope and stimulation of a stronger IFN-γ-producing HER 420–429-specific CD8+ T-cells. In contrast to linear HER-GLP-1, the branched HER-GLP-2 was still processed and presented in the presence of epoxomycin (Fig. 7B). This result shows that processing of branched HER-GLP-2 and presentation of HER420–429 epitope to CD8+ T cells is independent on the proteasome pathway. An opposite result was observed when monensin was used as the inhibitor (Fig. 7C). Monensin significantly inhibited the presentation of branched HER-GLP-2 but not the linear HER-GLP-1 indicating that at least partial processing of the branched HER-GLP-2 in the endosomal compartment and/or recycling through monensin-sensitive endocytic vesicles. Conversely, presentation of the linear HER-GLP-1 was not inhibited, suggesting that neither a monensin-sensitive endogenous pathway nor endosomal acidification were used during the processing of linear HER-GLP-1 construct.

Discussion

Glyco-lipopeptides, a form of lipid-tailed glyco-peptides, are currently under intense investigation as B- and T-cell based vaccine immunotherapy for many cancers [35,36,37,38,39]. In the present report, we describe the assembly, immunogenicity and antitumor efficacy of four-component HER-GLP vaccine construct (one CD4+ T-cell epitope, one OVA CD8+ T-cell epitope, one carbohydrate B-cell epitope, and a built-in palmitic acid adjuvant). The additional incorporation of a palmitic acid moiety in two different positions results in linear and branched constructs termed HER-GLP-1 and HER-GLP-2, respectively. We showed that both constructs are immunogenic. While the linear HER-GLP-1 induces more potent HER420–429-specific IFN-γ-producing CD8+ T-cell responses, the branched HER-GLP-2 promotes stronger tumor-specific IgG responses. Furthermore, although both constructs enter dendritic cells (DCs) via TLR2 and induced DCs maturation each construct appeared to be processed and presented to T cells differently. Accordingly, therapeutic immunization of mice with linear HER-GLP-1 versus branched HER-GLP-2 induced different levels of antitumor protective immunity. Thus, the position of the lipid moiety within synthetic GLP vaccine constructs greatly influence: (i) the magnitude of induced IgG and CD8+ T cell responses; (ii) the phenotypic and functional maturation of DCs; (iii) the cross-presentation pathway of the GLP constructs by DCs; and (iv) the level of therapeutic efficacy against established tumors.

This work demonstrate that the linear HER-GLP-1 construct induced higher magnitude of epitope-specific IFN-γ-producing CD8+ T-cell response while the branched HER-GLP-2 analogue preferentially induced stronger IgG antibody response. Besides

Figure 5. Relative uptake of linear HER-GLP-1; branched HER-GLP-2 and non-lipidated HER-GP molecules by bone marrow derived immature dendritic cells. (A) Primary cultures of bone marrow derived DC populations were incubated for 30 min at 37°C with Alexa Fluor 488-labeled HER-GLP-1, HER-GLP-2 or HER-GP at an equimolar concentration of 1 μM each. Left panel shows the dot plot representation of loaded HER-GLP and HER-GP constructs on CD11b/c+ cells and right panel shows the subsequent cytoplasmic localization of HER-GLP and HER-GPs by confocal microscopy. (B) Shows the uptake kinetics of HER-GLP and HER-GP constructs by CD11b/c+ cells following incubation at different time intervals as measured by the endocytosis assay uptake. Endocytosis assay uptake of Alexa Fluor 488-labeled HER-GP and HER-GLPs by DCs was determined for 120 min both at 4°C and 37°C. Subsequently, DCs were washed three times with phosphate-buffered saline/bovine serum albumin 1% and total uptake of Alexa Fluor 488-labeled was measured by FACS analyses and expressed by the difference in geometric mean that resulted from subtracting the values obtained at 4°C from the values obtained at 37°C. This formula determines the amount of HER-GP and HER-GLPs that is actively internalized. The results are representative of five experiments. doi:10.1371/journal.pone.0011216.g005
promoting a stronger CD8+ T cell response, the self-adjuvating linear HER-GLP-1 also induced higher-level phenotypic and functional maturation of dendritic cells. Finally, we established that the uptake of linear HER-GLP-1 construct did not follow the classical cross-presentation pathway as evidenced by the endosome-to-cytosol inhibition study, but rather appeared to take the proteasomal pathway. The molecular mechanisms that led to the exit of the epitopes from endosome to cytosol remain to be determined. To our surprise, the processing pathway of branched HER-GLP-2 was different from the linear HER-GLP-1 construct. The processing of branched HER-GLP-2 was not sensitive to epoxomicin suggesting that the branched HER-GLP-2 construct is processed through a proteasome-independent pathway. The branched HER-GLP-2 processing is, however, sensitive to both brefeldin A and monensin suggesting the processing is dependent on lysosomal acidification as well as on MHC class I recycling and transport from endosome to cell surface. To our knowledge, this is the first report that the position of the lipid moiety within a GLP vaccine construct greatly affects its cross-presentation pathway resulting in modulation of the magnitude of induced B and T-cell responses.

The processing of linear HER-GLP-1 is not sensitive to monensin suggesting that the pathway is independent of lysosomal acidification. As the processing of linear HER-GLP-1 is sensitive to both brefeldin A and epoxomicin it suggests that after uptake by DCs the construct was processed through proteasome mediated digestion followed by loading of the HER420-429 epitope on MHC class I molecules. In the endosomal system, the linear HER-GLP-1 may be protected from the predominant cleavage by the proteasome that would destroy the HER 420-429 epitope in the cytosol and may find a suitable enzyme for optimal generation and presentation of this epitope. However, the identity of this enzyme remains to be determined. Association with MHC class I molecules is possible in late endosomes, which were found to contain MHC class I molecules [40]. Alternatively, the linear HER-GLP-1 might also egress to the cytosol from a different, monensin-sensitive compartment than the branched HER-GLP-2 analogue and be digested by an enzymatic activity that is insensitive to epoxomicin. Finally, we do not exclude that the affinity of the chemical bond between the palmitic acid moiety and the peptide backbone might be slightly different during the synthesis of the linear HER-GLP-1 versus the branched HER-
GLP-2. This might play a role in determining how the GLP is taken-up and processed in DCs, as recently demonstrated using the palmitoylated encephalitogenic peptides of myelin proteolipid protein [41].

Our results demonstrated that both linear HER-GLP-1 and branched HER-GLP-2 constructs are taken up easily by dendritic cells (Fig. 5), and both constructs induced DCs maturation, albeit at different levels (Fig. 6A and 6B). Processing of both GLP constructs by DCs, which leads to different magnitudes of T cell stimulations (Fig. 3 and Fig. 7), appeared to involve binding/internalization via TLR-2 molecules. This is supported by our antibody blocking experiment, where blocking TLR-2, but not TLR-4, abrogated the presentation of CD8+ T cell epitope to HER420–429-specific IFN-γ-producing T cells (Fig. 6C). The position of the TLR-2 ligand palmitic acid appeared to influence the cross-presentation pathway of GLP vaccine constructs within DCs, and this might be a consequence of a difference in binding/internalization process via TLR-2. Recent study reported that peptides linked to TLR-2 ligand Pam3Cys of R-configuration (Pam(R)) lead to better activation of DCs compared to those with S-configuration (Pam(S)) [42,43]. Although both Pam(R) and Pam(S) epimers were internalized equally, the study concluded that the enhanced DC maturation is due to enhanced TLR-2 binding by the Pam(R)-conjugate in contrast to its Pam(S)-conjugate. Similarly, in case of linear and branched HER-GLP constructs, one cannot exclude the possibility of two different affinities of palmitic acid with TLR2, when placed in two different chemical conformations, cause differential uptake/processing in DCs [44]. Our results certainly show that the position of TLR-2 ligand palmitic acid, (i.e. linear or branched) greatly affects the uptake and the cross-presentation pathway of associated epitopes. In addition, the involvement of other TLRs and non-TLR receptors are not ruled out in the binding/internalization of linear HER-GLP-1 and branched HER-GLP-2 [45]. Another TLR-2 ligand lipoteichoic acid was reported be internalized independently from TLR2 [46]. Investigating the relative role of TLRs and other non-TLR receptors in: (i) binding/internalization; (ii) processing; and (iii) enhancing the immunogenicity and protective efficacy of GLPs is an important goal for future studies. Because each of the GLP constructs employed in the present study is molecularly defined, they can be labeled precisely on a single residue for future mechanistic evaluation.

Production of CD8+ T cell epitopes in APCs has been mostly documented as processing by the proteasome in the cytosol followed by TAP-mediated transport into the endoplasmic reticulum and association with nascent MHC class I molecules [40,47]. Among APCs, only DCs can prime naïve CD8+ T cells, and therefore they are required for primary immunization [40,48,49,50]. Dendritic cells have apparently evolved specific cross-presentation mechanisms allowing them to prime CD8+ T cells for exogenous Ags that are first internalized by macro-pinocytosis, phagocytosis, or receptor-mediated endocytosis [31,51,52,53]. Dendritic cells process these antigens and associate them with their MHC class I molecules [52]. Two major routes for cross-presentation have been described [31,53,54]: exit from the endocytic compartment and processing in the cytosol [55,56] and processing in the endosomal system and transfer of the peptides to recycling MHC class I molecules either in the endocytic pathway [57,58] or, after regurgitation, at the cell surface [59,60]. These mechanisms are essential for the cells to become sensitive to and develop tolerance to Ags that are not endogenously synthesized in DCs.

Cancer cells undergo significant changes in carbohydrate expression (aberrant glycosylation), and these alterations can be used as therapeutic targets (reviewed in [1]). Aberrant glycosylation of glycoproteins and glycolipids on tumor cells leads to expression of abnormal tumor associated carbohydrate antigens (TACAs) [61,62]. The most common TACA is Tn antigen (a precursor of Thomsen-Friedenreich or TF antigen), also known as GalNAc, which is α-linked to a serine or threonine residue (α-GalNAc-O-Ser/Thr) [63]. Tn is detected in up to 90% of human breast, ovary, and colon carcinomas [64]. However, the induction of IgG antibodies (Abs) against TACAs is much more difficult than eliciting similar Abs against pathogen associated carbohydrate antigens (reviewed in [1]). This is not surprising, because some TACAs are self-antigens and are consequently tolerated by the body.

Figure 7. Cross-presentation pathways of linear HER-GLP-1 and branched HER-GLP-2 vaccine constructs in dendritic cells. (A) Dendritic cells were pre-incubated with brefeldin A for 1 h, followed by the addition of linear HER-GLP-1, branched HER-GLP-2 or non-lipidated HER-GP construct (open bars). As a positive control, DCs were left untreated with antigen-processing inhibitors but were pulsed with linear HER-GLP-1, branched HER-GLP-2 or parent non-lipidated HER-GP (open bars). After overnight incubation, DCs were then washed and added to the HER420–429-specific CD8+ T cells for additional 5 hrs. IFN-γ produced by HER420–429-specific CD8+ T cells was tested by ELISpot assay. Panel (B) and (C) represents identical experiments conducted in the presence of epoxomycin and monensin inhibitors, as described in Materials and Methods. Results are representative of three independent experiments. doi:10.1371/journal.pone.0011216.g007
immunogenic vaccines [3,65,66,67,68]. The shedding of TACAs by growing tumors exacerbates this tolerance [3,65,70]. However, under appropriate conditions, Tn can induce tumor-specific IgG in both mice and non-human primates [29]. Overall, the rates of Tn expression showed statistically significant differences between healthy and tumorous or transitional tissues [71,72]. Accordingly, several Tn-based vaccines and immunotherapies that passed clinical trials showed no major side effects [73]. This raises the hope that aberrantly glycosylated TACAs can be used as a specific target for humoral-mediated cancer vaccines.

The studies reported here show that four-component HERGLP vaccine molecules elicit robust IgG antibody response. The position of the lipid moiety significantly affects the level of IgG production and the optimal binding to breast cancer cell lines expressing carbohydrate antigens on their surface [20]. Previous studies demonstrated that lipid-tailed peptides promote a T cell-independent activation and maturation of B-cells via TLR-2 and increased the frequency of IgG secreting B-cells [74]. Although B-cells expressing TLR-2 are potential targets, very little is known about the effect of GLP on B-cells and especially on their potential for inducing carbohydrate-specific Ab response. Our findings demonstrate that the branched HER-GLP-2 promoted strong Tn carbohydrate-specific IgG response. The same Abs bind to intact Tn expressing breast tumor cells, suggesting that biologically relevant specificities were produced. Although we found that PADRE-specific CD4+ T cell proliferative responses were not affected by the position of the lipid moiety (not shown), this does not rule out the alteration of balance between Th1/Th2 helper cytokines. Why the branched HER-GLP-2 skewed towards stronger RAFT-specific IgG production than linear HER-GLP-1 remains to be determined. Nevertheless, our findings are important, particularly in view of the problems associated with large carrier proteins, such as tetanus toxoid or diphtheria toxoid, often used to deliver weakly antigenic molecules such as carbohydrates [75]. In addition, “booster” injections are often required for conversion of the initial, transient IgM response to a strong, durable IgG response. However, Abs responses directed against the vaccine carrier have been shown, in some cases, to negatively affect the booster response to the vaccine Ag [76]. The use of a totally synthetic GLP carrier molecule capable of inducing vigorous helper T cells, but potentially less readily recognized by Abs might be, in this respect, of significant interest. Together these data support our belief that GLP should be considered as an alternative to more complex carriers for use in prophylaxis and therapeutic cancer vaccines.

The cellular and molecular mechanisms underlying the immunogenicity of HER-GLP remain to be fully elucidated. Our data indicate that the lipid moiety is a crucial factor since the immunogenicity of HER-GLP is superior to its non-lipidated HER-GP analog. Non-lipidated HER-GP failed to cross cell membranes, failed to induce maturation of DCs, and failed to present the HER420–429 peptide epitope to CD8+ T cells. In addition, vaccine constructs missing any individual component out of the four did not produce any immune responses suggesting that it is crucial to have all the components linked together in one molecule. In contrast, lipid-tailed HER-GLP vaccine molecules easily attach and cross the cell membrane of DCs and drive their maturation of DCs. In contrast, the lipid-tailed HER-GLPs easily attached and crossed the cell membrane of DCs and drive their maturation. The linear HER-GLP-1 construct was taken up more efficiently by DCs than the branched HER-GLP-2 analog. The present report thus extends previous findings, by showing that in vitro incubation of immature DCs with HER-GLP molecules interacts with TLR-2 [37] and increased cell surface expression of MHC class II and CD80/CD86 co-stimulatory markers indicating DC maturation. Untreated immature DCs and lipid-moiety treated DCs were used as negative controls. Under similar conditions, there was no up-regulation of MHC class II and costimulatory molecules or production of IL-12 or TNF-α following incubation of immature DCs with either the parental non-lipidated HER-GP alone or the lipid moiety alone (Fig. 6A and 6B, p<0.05). Non-lipidated HER-GP failed to cross cell membranes and failed to induce phenotypic and function maturation of DCs. In contrast, the lipid-tailed HER-GLPs easily attached and crossed the cell membrane of DCs and drive their maturation. The linear HER-GLP-1 construct was taken up more efficiently by DCs than the branched HER-GLP-2 analog. The present report thus extends previous findings, by showing that in vitro incubation of immature DCs with HER-GLP molecules interacts with TLR-2 [37] and increased cell surface expression of MHC class II and CD80/CD86 co-stimulatory molecules resulting in mature DCs producing high levels of IL-12p35 and TNF-α cytokines [1,21,24]. Together, these results indicated that covalent linkage to the lipid moiety is required for DCs maturation. This suggests that, as a mechanism behind the immunogenicity of GLPs, the lipid moiety likely exerts its adjuvant effect by interacting and stimulating DCs.

Synthetic cancer vaccines, offer safety, reliability and cost advantages over traditional methods (e.g. live vectors, tumor cells-APC fusions, genetic immunization), but formidable challenges still confront their development [21,88,89]. Among these is the requirement for external immuno-adjuvant, which is critical for the immunogenicity and protective efficacy of synthetic vaccines [90,91]. An ideal adjuvant should rescue and increase the immune response against tumors, with acceptable toxicity and safety even for those immuno-compromised cancer patients [21,90]. While several different adjuvants are effective in pre-clinical studies, the aluminum-based salt (Alum) is currently the only licensed adjuvant [92,93] for clinical application. Although “alum” is able to induce a strong antibody (Th2 type) response, it has little capacity to stimulate cellular (Th1 type) immune responses, which are important for protection against many cancers [8,94]. Therefore, safe and effective self-adjuvanting molecules are highly desired [3,21]. These self-adjuvanting cancer vaccine molecules should be more potent but less toxic than external adjuvants [95,96]. Owing to the limited success of many vaccines in the clinic, attempts are being made to improve the safety and efficacy of vaccine formulations, and to define new adjuvants and antigen delivery systems. However, the development of new cancer adjuvants as well as the improvement of efficacy and safety of existing adjuvants...
still lags far behind [92,97]. We have previously shown that the “new generation” four-component, self-adjuvanting GLP vaccine molecule, might offer a compromise between highly toxic adjuvants and no chemical adjuvants at all, while inducing a strong protective immunity [1,21,24]. In this study we showed the protective efficacy of a GLP vaccine molecule and demonstrated that the therapeutic efficacy of HER-GLP is affected by the position of the lipid moiety.

Bearing in mind the particular constraints for a prospective human vaccine, the present study designed prototype self-adjuvanting HER-GLPs vaccines and demonstrated their safety, immunogenicity and protective efficacy in a mouse tumor model. Molecularly defined epitope-based vaccines capable of inducing anti-tumor protective immunity, in a manner compatible with human delivery, are limited. Few molecules achieve this target without being delivered with potentially toxic external immunoadjuvants. It is important to note that even moderate levels of IFN-γ-producing CD8+ T cell responses were induced by HER-GLP vaccine constructs (Fig. 3), they were sufficient to control tumor progression (Fig. 4A) and to protect against death (Fig. 4B). This suggests that the quality, rather than the quantity, of the CD8 T cell responses was more crucial in protecting against cancer in this mouse model. We have previously shown that immunization with MHC I-restricted CTL peptides+helper peptides in IFA induced higher magnitude of CTL responses compared to when the same MHC I-restricted CTL peptides+helper peptides are attached to a lipid moiety (i.e. lipopeptides), delivered without external immuno-adjuvant [9,13]. However, unlike external toxic Freund’s adjuvants [98], the TLR-2 ligand palmitic acid, has been used as a built-in immuno-adjuvant, and was safe and immunogenic in both animals and humans [15,14,16,17,85,99,100]. Because several lipid-tailed peptide vaccine candidates have been recently employed in clinical trials [101,102,103], we expect that, once a potent lipid-tailed GLP cancer vaccine is validated in preclinical animal studies, the move to a clinical trial should be straightforward.

A synthetic liposomal ErbB2/HER2 peptide-based vaccine construct with the combination of CD8+ and CD4+ epitopes has been recently reported to induce prophylactic and therapeutic protection in mice [104] but concerns remain about its potentially toxic adjuvants. Those studies prompted us to construct and test self-adjuvanting HER-GLP vaccines. Kieber-Emmons and co-workers showed Pam3CSS moiety serves as a built-in adjuvant and enhances tumor-specific Abs [22]. Later a three-component GLP vaccine by Boons and coworkers (a three palmitic acid Pam3CSSK2 moiety, a CD4+ and a B-Cell epitope) showed induction of strong tumor-specific IgG responses [3]. To our knowledge, we are the first to report self-adjuvanting four-component HER-GLP vaccine molecules. The previously reported complex Pam3CSSK2 and Pam3CSS molecules have a spontaneous tendency to form stable aggregates, making the synthesis, purification and solubility of Pam3CSS-tailed HER-GP extremely difficult. In contrast, our HER-GLP vaccine constructs were synthesized using the mono-palmitoyl strategy which is relatively simple to produce and easy to purify under GMP conditions.

In summary, we have demonstrated that fully synthetic self-adjuvanting linear and branched HER-GLP vaccine molecules follow different cross-presentation pathways in DCs and generate different magnitudes of B- and CD8+ T-cell responses. The position the lipid moiety in the HER-GLP construct profoundly affects phenotypic and functional maturation of DCs, the processing of GLP molecules and its cross-presentation in DCs; as well as the magnitude of IgG and IFN-γ producing CD8+ T cell responses. Finally, the position of the lipid moiety also affected the strength of immunotherapeutic efficacy induced by the GLP vaccine constructs. The advantage and relative ease of making these self-adjuvanting GLP cancer vaccines will greatly facilitate the production of GLP cancer vaccines for large-scale clinical trials. Their clinical success will depend in great measure on selection of the appropriate human TAAs and TACAs epitopes.

Materials and Methods

Peptide, glyco-peptide and glyco-lipopeptides synthesis

Peptides. Protected amino acids and Fmoc-Gly-Sarrin resin were obtained from Advanced ChemTech Europe (Brussels, Belgium), Bachem Biochimie SARL (Vosins-Les-Bretonneux, France) and France Biochem S.A. (Meudon, France). PyBOP was purchased from France Biochem and other reagents were obtained from either Aldrich (Saint Quentin Fallavier, France) or Acros (Noisy-Le-Grand, France). Reverse phase HPLC analyses were performed on Waters equipment. The analytical (Nucleosil 120 Å 3 mm C18 particles, 30×4.6 mm) was operated at 1.3 mL/min and the preparative (Delta-Pak 300 Å 15 mm C18 particles, 200×25 mm²) for glycopeptides and Discovery Bio Wide Pore C3, 25×10 mm) was performed at 12 mL/min with UV monitoring at 214 nm and 250 nm using a linear A-B gradient (buffer A: 0.09% CF3CO2H in water; buffer B: 0.09% CF3CO2H in 90% acetonitrile). Mass spectra were obtained by electron spray ionization (ES-MS) on a VG Platform II in the positive mode.

Glyco-peptides. Compound 1 [21,24] (14 mg, 5.89 μmol) was dissolved in a degassed mixture of sodium acetate buffer 25 mM pH 5 and isopropanol (6 mL, 1/1). The peptide HER420–429-PADRE-Cys2 (16.6 mg, 5.89 μmol) was added and the solution was stirred until completeness of the reaction (checked by analytical RP-HPLC). After 2 h, the crude yellow reaction mixture was purified by semi-preparative RP-HPLC (C18 column, linear gradient: 95:5 to 0:100 A:B in 30 min, Rf = 13.8 min) to obtain a lyophilized powder corresponding the pure glycopeptide HER-GP (8 mg, 27% yield). Analytical data: Rf = 8.2 min (C18 analytical column, linear gradient: 95:5 to 0:100 A:B in 15 min); ES-MS (positive mode) calculated for C221H361N58O72S2 5043.6, found 5043.4.

Linear glyco-lipopeptide (HER-GLP-1). A similar procedure was followed from 1 (15 mg, 6.31 μmol) and the lipopeptide PAM-HER420–429-PADRE-Cys2 (19.3 mg, 6.31 μmol) for the synthesis of the glycopeptide-HER-GLP-1. After purification by semi-preparative RP-HPLC (C5 column, linear gradient: 95:5 to 0:100 A:B in 30 min, Rf = 22.5 min) the glycopeptide-HER-GLP-1 was obtained as pure lyophilized powder (6 mg, 18% yield). Analytical data: Rf = 11.8 min (C18 analytical column, linear gradient: 95:5 to 0:100 A:B in 15 min), ES-MS (positive mode) calculated for C237H391N58O73S2 5281.8, found 5282.8.

Branched glyco-lipopeptide (HER-GLP-2). A similar procedure was followed from 1 (15 mg, 6.31 μmol) and the lipopeptide PAM-HER420–429-PADRE-Cys2 (19.3 mg, 6.31 μmol) for the synthesis of the glycopeptide-HER-GLP-2. After purification by semi-preparative RP-HPLC (C5 column, linear gradient: 95:5 to 0:100 A:B in 30 min, Rf = 22.5 min) the glycopeptide-HER-GLP-1 was obtained as pure lyophilized powder (5 mg, 15% yield). Analytical data: Rf = 11.3 min (C18 analytical column, linear gradient: 95:5 to 0:100 A:B in 15 min), ES-MS (positive mode) calculated for C243H403N59O73S2 5308.8, found 5308.9.

Immunization and serum preparation

Female B10.D1 mice 4 to 5 weeks old were purchased from Jackson Laboratory (Bar Harbor, ME) and immunized subcutaneously at the base of the tail either with (i) linear HER-GLP-1
(50 μM/mouse in 100 μl of PBS) or with (ii) branched HER-GLP-2 (50 μM/mouse in 100 μl of PBS) or with (iii) HER- (50 μM/mouse in 100 μl of PBS) or (iv) PBS alone, three times at 14 day intervals. Ten days after the last immunization, mice were bled from the ocular venus plexus. Serum were collected by centrifugation for 5 min at 1250 RPM and stored at –80°C.

Antibodies
Streptavidin coated 96-well ELISA plates were purchased from NUNC. MultiScreen HTS™ IP plates for ELISpot assays were purchased from Millipore. Goat anti-mouse IgG-HRP was purchased from Chemicon. Goat anti-mouse IgG-FITC was purchased from Sigma. TMB substrate reagent set was purchased from BD Biosciences.

ELISA and ELISpot assay
Immune and non-immune mouse sera were tested for anti-RAFT antibodies by using direct ELISA, as we recently described [21,82]. ELISpot assay was performed by using cytokine ELISpot pair kit from BD PharMingen, San Diego, CA, as we recently described [21].

Cell lines
MCF-7, the human breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA) and grown to 90% confluence in Modified Dulbecco’s medium, as we recently reported [5,6,21,105]. The NT2 neu-expressing tumor line derived from spontaneously arising mammary tumors excised from neu-N mice was used in experiment of tumor growth inhibition [106]. NT2 cells express stable neu and MHC class I, as previously described [106].

Generation of DCs
Bone marrow-derived DCs were generated using a modified version of our previously described protocol [85]. Briefly, a total of 2×10^6 bone marrow cells were cultured in tissue dishes containing 10 ml of RPMI 1640 supplemented with 2 mM glutamine, 1% nonessential amino acids (Gibco-BRL), 10% FCS, 50 ng/ml of GM-CSF and 50 ng/ml IL-4 (PeproTech Inc). Cells were feed 10 ml of RPMI 1640 supplemented with 2 mM glutamine, 1% albumin and total uptake of Alexa Fluor 488-labeled was measured by FACS. The data are expressed by the difference in geometric mean after subtracting the values obtained at 4°C from the values obtained at 37°C. This formula determines the amount of HER-GP and HER-GLPs that is actively internalized.

Flow Cytometry
200 μl of 4×10^5 cancer cells were incubated with 10 μl of (1:250 dilution) immunomodulating or non-immune serum for 30 min. at 4°C in FACS staining buffer (PBS containing 5% fetal calf serum and 0.1% sodium azide). Cells were washed two times with buffer followed by centrifugation for 5 min at 1250 RPM and 5°C after each wash. Cell pellets were suspended in 200 μl of FACS staining buffer and incubated with 10 μl of (undiluted) secondary antibody (anti-mouse IgG-FITC) for 30 min. at 4°C. Cells were washed two times as above. The final cell pellets were suspended in 250 μl of FACS staining buffer and data acquired immediately on a FACSscan flow cytometer (Becton Dickinson, Mountain View, CA, USA) and analyzed with CellQuest software (Becton Dickinson).

Blocking TLR-2 receptors on DCs
Blocking of TLR-2 receptors on DCs were performed on day 4 after the initiation of the culture from bone marrow derived cells, as previously described [85]. Anti-TLR-2 mAb (Bioscience) or an IgG1 isotype control mAb (10 μg/ml) was added to DCs 30 min before the addition of the peptide or lipopeptide. Cells were harvested 48 h later and counted directly in ELISpot Assay.

Monensin antigen-processing inhibitors
Dendritic cells were first treated with brefeldin A, Epoxomicyn or Monensin, followed by the addition of either linear HER-GLP-1, branched HER-GLP-2 or parent non-lipidated HER-GP construct and incubated overnight with HER_{420-429}-specific CD8^+ T cells. When used, brefeldin A (Sigma) or epoxomicyn (Alexis Biochemicals) was added 1 h before the addition of linear HER-GLP-1, branched HER-GLP-2 or parent non-lipidated HER-GP constructs at concentrations of 10 μg/ml and 10 μM, respectively, and then diluted at 2 μg/ml and 2 μM for the overnight incubation [26,27]. Monensin (Sigma) was added only 10 min after linear HER-GLP-1, branched HER-GLP-2 or parent non-lipidated HER-GP addition (in an attempt to avoid preventing their endocytosis) at a concentration of 50 μM. In all cases, after overnight incubation, DCs were then washed and added to the HER_{420-429}-specific CD8^+ T cells (ratio, 1:1) in the presence of brefeldin A (10 μg/ml) for 5 h at 37°C. CD8^+ T cells incubated in the presence of non-treated but pulsed DCs were used as positive controls. After inhibition CD8^+ T cells were assessed in ELISpot for IFN-γ production as indicated above.

Tumor immunotherapy
Ten mice in each experimental group were inoculated s.c. in the upper back with 1×10^6 NT2 cells/mouse. Local tumor diameter was measured with calipers. Starting 8–10 days later, when the
tumor reached 3–4 mm in diameter, mice were immunized sc. four times at 7-day intervals with GLP-1 and/or GLP-2 or control PBS (Mock) on days 0, 7 and 14 and 21, as described above. Tumor diameter and survival were recorded. The length, width and height of each tumor were measured using a digital slide caliper. Tumor volume was calculated by the formula: \( V = \frac{4}{3} \pi \times \text{length} \times \text{width} \times \text{height} \).

Statistical analysis

Statistical differences in tumor sizes between groups of mice were determined by one-way ANOVA. Significance of survival plots was done with Kaplan-Meier survival platform. For both analyses, we used the JMP statistics software (SAS Institute).

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