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ARTICLE

GLYCODENDROPEPTIDES STIMULATE DENDRITIC CELL MATURATION AND T CELL PROLIFERATION. A POTENTIAL INFLUENZA A VIRUS IMMUNOTHERAPY.

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Ainhoa Mascaraque,^a Wioleta Kowalczyk,^b Tahia Fernández,^c Francisca Palomares,^c
Cristobalina Mayorga,^{*c} David Andreu^{*b} and Javier Rojo^{*a}

[a] Glycosystems Laboratory, Instituto de Investigaciones Químicas (IIQ), CSIC - Universidad de Sevilla, Américo Vespucio 49, 41092 Sevilla, Spain; Fax: +34-954460165, E-mail: javier.rojo@iiq.csic.es; [b] Department of Experimental and Health Sciences, Universitat Pompeu Fabra, 08003 Barcelona, Spain; Fax: +34-933160901, E-mail: david.andreu@upf.edu; [c] Laboratory of Research, UGC de Alergología, IBIMA-Hospital Regional Universitario de Málaga-UMA, 29009 Málaga; Fax: +34-951290203, E-mail: mayorga.lina@gmail.com

Mannosylation facilitates the uptake and the internalization of immunogenic peptides by antigen-processing cells expressing mannose receptors at their surface, such as DC-SIGN, a lectin that plays a key role in the immune response against different pathogens. This internalization, processing and subsequent MHC presentation may result in a strong T-cell stimulation. Here, we hypothesized that combining mannose glycodendrons with multivalent presentation of peptide epitopes in likewise dendron format would yield hybrid constructs, named *glycodendropeptides* (GDPs), with the capacity to enhance peptide immunogenicity, hence providing a novel and versatile platform for applications in immunotherapy. Thus, GDPs of different valencies displaying the NP₃₆₆₋₃₇₄ epitope, a conserved sequence from the influenza A virus nucleoprotein (NP), have been built by two *click chemistry*-based methodologies and assessed as potential flu vaccine candidates. Preliminary evaluation of the ability of these constructs to stimulate dendritic cell maturation and lymphocyte proliferation was promising, showing the highest-functionalized NP₃₆₆₋₃₇₄ GDPs as inducing the strongest immunostimulatory effect.

Introduction

The biological effect of peptide or protein mannosylation,¹ which induces a receptor dependent internalization process in dendritic cells (DCs) and ensuing strong T-cell stimulation, constitutes an interesting tool for developing novel strategies in the vaccine field.²

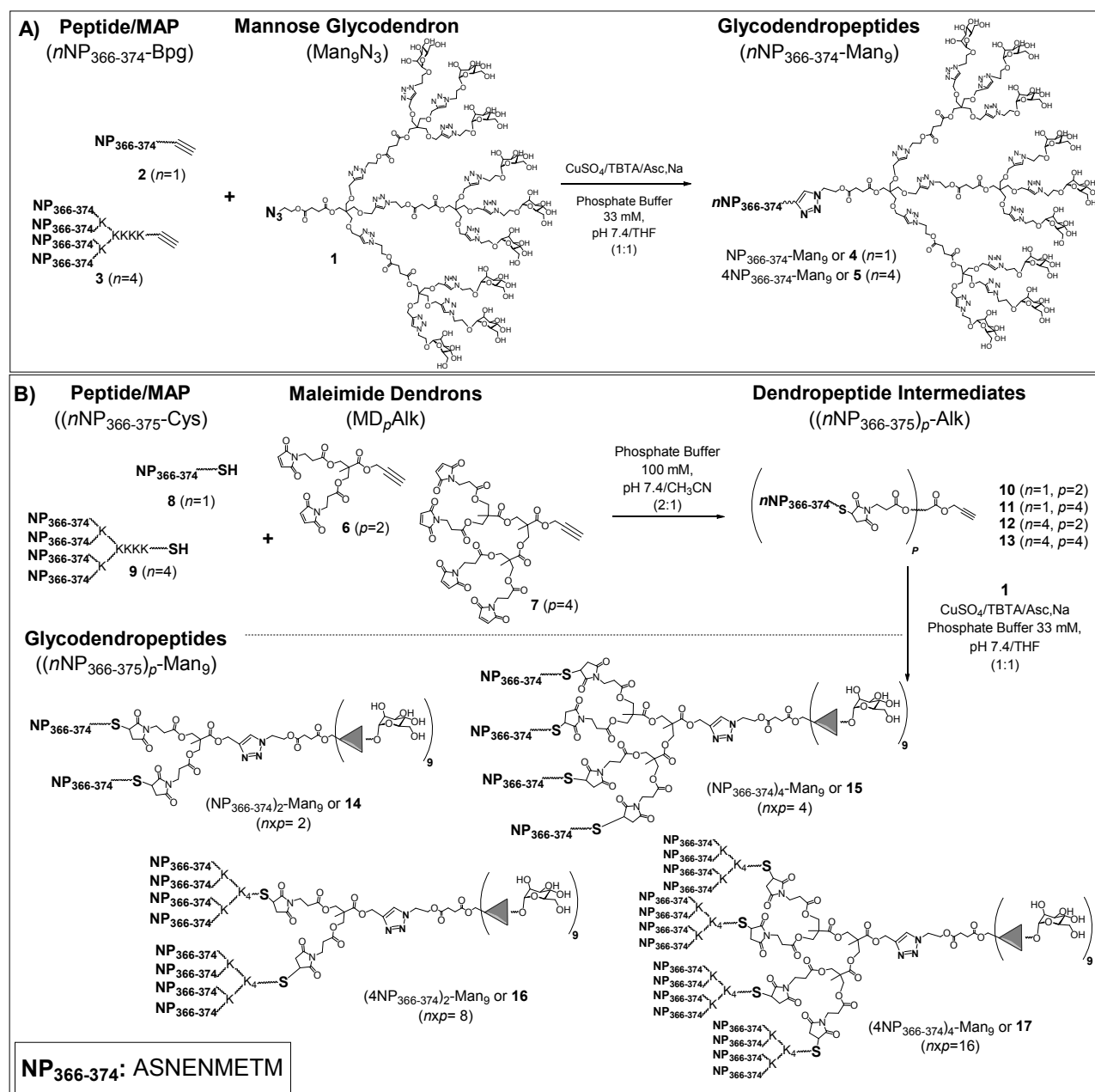
Compared to entire protein immunogens, peptides show clear advantages such as stringent chemical definition, fast and efficient large scale production, or simple (cold chain-free) transport and storage.³ These advantages, however, can be offset by the relatively poor immunogenicity of peptides, a limitation often overcome by multiple presentation of the peptide antigen, such as the dendrimer systems pioneered by Tam under the name of Multiple Antigen Peptides (MAP).⁴

Our group has recently described hybrid constructs named *glycodendropeptides* (GDPs), where the multivalency of peptide epitopes in a MAP format combined with the improved uptake facilitated by mannosylation may lead to enhanced immunogenicity.⁵ Specifically, mannosylation would induce MAP internalization into DCs through interactions with the DC-SIGN (Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Non-integrin) receptor,⁶ a lectin expressed at the surface of immature DCs (imDCs). This receptor is involved in the internalization of highly mannosylated antigens to be processed and presented to T cells to elicit an immune response.⁷ Recently, we have shown that a dendron functionalized with nine copies of mannose (Man) has the ability to interact, in a sugar dependent manner, with DC-SIGN and become

internalized into DCs.⁸ In this context, the nonavalent glycodendron **1** (Scheme 1) was selected as the carbohydrate moiety of the GDPs used in the present study, although other Man valencies could be easily examined as well.

Two different synthetic approaches (Methods A and B, Scheme 1)⁵ provide efficient and convergent routes to chemically well-defined GDPs that combine, in a single platform, multiple carbohydrate and peptide epitopes. The modularity and versatility of these routes allow a variety of biological applications where both carbohydrate and peptide, as well as their respective valencies, can be selected at will.

Influenza A is one of the most important respiratory pathogens worldwide, being vaccination the widely accepted way to control the disease.⁹ However, in contrast to other pathogens that require vaccination only few times in a lifetime, influenza vaccines have to be administered yearly. This is due to the remarkable mutation rates –and ensuing antigenic variability– of the two main envelope proteins, hemagglutinin (HA) and neuraminidase (NA), that up to now constitute the cornerstone of practically all influenza vaccines. It has been argued, however, that viral proteins with much lower yearly mutation rates could serve as better immunogens in a future universal flu vaccine.¹⁰ One such highly conserved protein is the nucleoprotein (NP) that encapsidates the viral RNA; in particular, the nine-residue epitope NP₃₆₆₋₃₇₄ has been proposed as a potential candidate immunogen for a universal influenza A vaccine.¹⁰⁻¹¹



Scheme 1. Synthetic routes to *glycodendropeptides* (GDPs): A) Method A; B) Method B, (where n = number of peptides; and p = number of maleimides. In Method B, final GDPs would be functionalized with $n \times p$ copies of peptide).

With this background, we decided to incorporate the NP₃₆₆₋₃₇₄ epitope (ASNENMETM) into novel GDPs that might serve as universal flu vaccine candidates. To this end, GDPs 4-5 and 14-17 were synthesized by the formerly mentioned Methods A and B (Scheme 1).⁵ Both approaches rely on two varieties of *click chemistry* reactions, namely the Huisgen's dipolar Cu^I-catalyzed azide-alkyne cycloaddition (CuAAC)¹² and the thiol-ene addition of thiols to maleimides.¹³ The GDP systems thus generated, providing multiple presentation of a conserved and relevant flu epitope, as well of Man residues with expected DC internalization capability, constitute novel and versatile platforms with enhanced immunogenic profiles that might eventually find use as synthetic influenza A vaccines.

Results and Discussion

The preparation of GDPs by the methods in Scheme 1 required various building blocks (the glycodendron moiety, two maleimide scaffolds and the peptidic components-peptides and MAPs-) that were prepared as previously described.⁵ Briefly, the nonavalent Man glycodendron 1 was conveniently built in a convergent process that used pentaerythritol as core and 1,3-dipolar cycloadditions for extension; maleimide dendrons 6 and 7 were derived from bis-2,2'-hydroxymethyl propionic acid (bis-MPA)⁵, and the single and multiple peptide modules (2, 3, 8, and 9) were made by Fmoc solid phase synthesis (SPPS).^{4, 14} All peptides and MAPs were C-terminal carboxamides of either a bishomopropargylglycine (Bpg) residue (2-3), providing the alkyne functionality required for CuAAC (Scheme

1A), or a Cys residue (8-9) supplying the thiol group for thiol-ene ligation (Scheme 1B). In the MAP syntheses, spacers reported to enhance the flexibility of the construct and the quality of the final product, were incorporated at all branching points.¹⁵ For MAP 3, the spacer was 6-aminohexanoic acid (Ahx), whereas 8-amino-3,6-dioxaoctanoic acid (O₂Oc) was selected for 9. All peptide components were obtained in homogeneous (>95% HPLC) form, with MS data consistent with theoretical values (Table S1, Supporting Information).

To access GDPs by method A (Scheme 1), peptide 2 or MAP 3 were directly conjugated with glycodendron 1 by a single CuAAC reaction. The best results were obtained with 1 equiv. of CuSO₄·5H₂O, 2 equiv. of TBTA (Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine) and 4 equiv. of sodium ascorbate at room temperature in a 1:1 (v/v) THF/phosphate buffer (33 mM, pH 7.4). The reproducibility and reliability of the conditions enabled the preparation of the final GDPs 4 and 5 (with 9:1 and 9:4 mannose-to-peptide ratios, respectively), with almost quantitative conversions from starting materials to final products and in a monodisperse way, as determined by both analytical RP-HPLC and MS (Table S1, Supporting Information). The relatively small synthesis scale translated in only moderate yields of HPLC-purified products, typically in the 35–50% range.

On the other hand, method B (Scheme 1) required two synthetic steps to reach the target products. In this approach, maleimide dendrons 6 and 7 served both to multiply peptide valency and, by way of the alkyne group at their focal positions, to enable conjugation to glycodendron moiety 1. In the first step, peptide 8 or MAP 9, either one featuring a C-terminal Cys, were chemoselectively ligated with di-(6) or tetravalent (7) dendrons by thiol-ene addition in a 1:2 (v/v) CH₃CN/phosphate buffer (100 mM, pH 7.4). The resulting dendropeptide intermediates 10-13 were obtained with almost quantitative conversions and in moderate yields after RP-HPLC purification. This first step allowed access to a dendropeptide (13) with up to 16 peptide copies. Although globally its synthesis involved long reaction times and moderate conversion rates, the end-product was again shown to be clearly monodisperse by analytical RP-HPLC and MS (Table S1, Supporting Information).

Once dendropeptide intermediates (10-13) were available, the corresponding GDPs (14-17) were readily obtained by CuAAC-mediated conjugation with glycodendron 1 under the same conditions set up for method A (Scheme 1). By means of this strategy, a GDP with a 9:16 Man/peptide ratio (17) was obtained which, to the best of our knowledge, is the largest multivalent and mannosylated peptide system thus far reported, again obtained as a monodisperse entity as proven by both HPLC and MS (Table S1, Supporting Information).

With all the above compounds in hand, we evaluated their effect on specialized cells of the immune system, such as DCs and lymphocytes. To this end, DCs were incubated with GDPs at different concentrations and the expression of maturation and costimulatory markers (HLA-DR, CD80, CD83 and CD86) was assessed. The results obtained in these experiments indicated that, at a given GDP concentration of 1 μM, the expression patterns of HLA-DR, CD80 or CD86 markers were maintained almost constant regardless of the multivalency of the NP₃₆₆₋₃₇₄ moiety. In contrast, an increase in CD83 expression was observed with the (4NP₃₆₆₋₃₇₄)₄-Man₉ GDP (17), with the highest peptide multiplicity (16 copies), one log above the responses observed for any other compound (p<0.005 for all) (Figure 1).

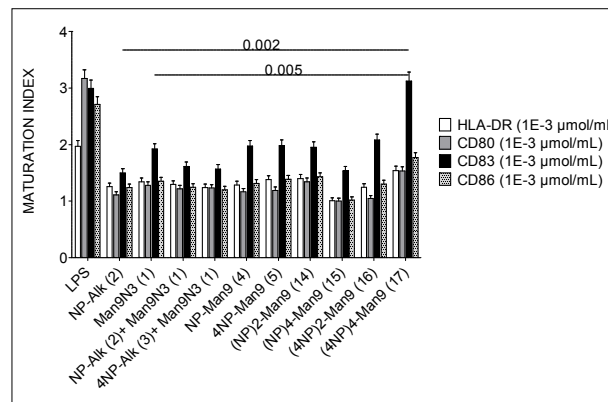


Figure 1. Mean and standard deviation of the maturation index of DCs expressing HLA-DR, CD80, CD83 and CD86 when compared with their respective expression in imDCs. The expression of markers was measured after 48 h incubation with the GDPs at 10⁻³ μmol/mL or LPS at 1 mg/mL as positive control. Cells were stained using specific antibodies against each marker and analysed by flow cytometry.

Such an increase in (4NP₃₆₆₋₃₇₄)₄-Man₉-induced CD83 expression in DCs may have important implications from an immunological point of view, since different studies have demonstrated that CD83, as the characteristic marker of full DC maturation,¹⁶ hence involved in T-cell-mediated immune response,¹⁷ is essential for DC biology. The effect observed with compound 17 might overcome the down-modulation in CD83 expression produced upon infection by several viruses, which reduces the ability of infected DCs to stimulate T cells.¹⁸

The proliferative responses of diverse lymphocyte subpopulations after incubation with GDPs at different concentrations were studied by analysing CD3, CD4, CD8 and CD19 expression (Figures 2 and 3).

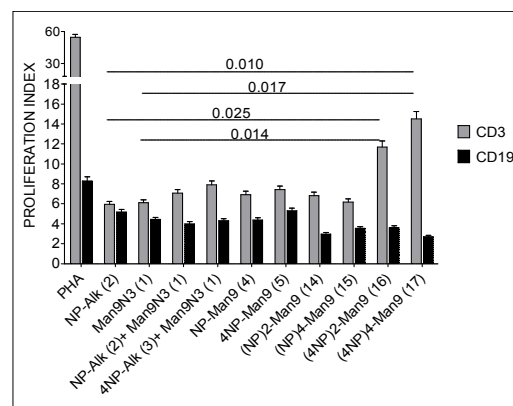


Figure 2. Proliferation index (PI) of CD3 and CD19 lymphocytes after treatment with different GDPs (1 μM) or PHA (Phytohemagglutinin)(10 μg/mL) as positive proliferative control for 6 days at 37 °C including imDCs as antigen presenting cells.

In the presence of NP₃₆₆₋₃₇₄-displaying compounds at a fixed 1 μM concentration, an increase in T lymphocyte (CD3) proliferative response with peptide multiplicity is observed, with significant highest values found for (4NP₃₆₆₋₃₇₄)₂-Man₉ (16) and (4NP₃₆₆₋₃₇₄)₄-Man₉ (17). Conversely, B lymphocyte (CD19) proliferation decreases with multivalency, although these differences are not significant (Figure 2).

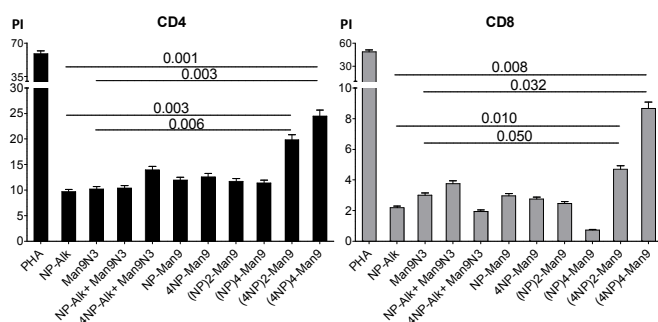


Figure 3. Proliferation index (PI) of CD4 and CD8 lymphocytes after treatment with different GDPs (1 μ M) or PHA (10 μ g/mL) as positive proliferative control for 6 days at 37 $^{\circ}$ C including imDCs as antigen presenting cells.

It is important to remind at this point that, during some infection processes, the immunological responses induced by viral agents lead to T cell proliferation, and specifically to CD8 T cells. In the present case, our data show a significant increase ($p < 0.05$) in the proliferative response of both CD4 and CD8 subsets of T lymphocytes (Figure 3). As observed with CD83 expression, the highest proliferation index (PI) was observed for the compound with the highest peptide multiplicity, namely (4NP)₃₆₆₋₃₇₄4-Man₉ (**17**). This could be explained by the fact that, as demonstrated by others, CD83 has an important effect in regulating activation of functional T cells.¹⁹

Although a much higher PI was observed for CD4 T cells after incubation with (4NP)₃₆₆₋₃₇₄4-Man₉ (**17**) (Figure 3), the proliferative response seems to be more specific for CD8 T cells, since relatively higher increases were found for this compound when compared to those induced by either peptide or glycodendron alone. These findings agree with those from other authors who have shown that CD83 expression directly correlates with an ability to induce specific cytotoxic T lymphocyte proliferation.^{18a} Taken together, our results from maturation analysis and proliferative response lead us to conclude that (4NP)₃₆₆₋₃₇₄4-Man₉ (**17**) could be a good vaccine candidate, given its effectiveness in inducing complete DC maturation (CD83 increase), hence a subsequent increase in specific cytotoxic T lymphocyte proliferation. Further studies to characterize the immunological response of these T cell subpopulations are being carried out.

Experimental Section

Materials and Methods: Fmoc-protected amino acids were from Iris Biotech (Marktredwitz, Germany). Fmoc-Rink-amide ChemMatrix resin, 2-(1H-benzotriazol-1-yl)-1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*-hydroxybenzotriazole (HOBt) were from Matrix Innovation (Montreal, Canada). All other reagents were of the highest quality commercially available from Sigma-Aldrich (Madrid, Spain). All solvents, including HPLC-grade CH₃CN, and peptide synthesis-grade DMF, CH₂Cl₂, DIEA and TFA were from Carlo Erba-SDS (Sabadell, Spain). Non-peptide compounds were purified by flash chromatography using medium or fine silica gel or by Sephadex LH20 or G25 (GE Healthcare, Barcelona, Spain) gel filtration. Thin layer chromatography (TLC) was carried out with pre-coated F254 silica gel plates (Merck, Darmstadt, Germany). Flash chromatography was carried out with silica gel 60 (230–400 mesh) (Macherey-Nagel, Düren, Germany). TLC reaction monitoring was performed using potassium permanganate, phosphomolybdic acid, 10% sulfuric acid in methanol or anisaldehyde as development reagents. Analytical

HPLC was carried out on 4.6 \times 50 mm, 3 μ m C18 or C8 columns (Phenomenex, Torrance, CA) with a LC-2010A system (Shimadzu, Kyoto, Japan). Solvents A and B were 0.045% and 0.036% (v/v) TFA in H₂O and CH₃CN, respectively. Elution was with linear gradients of solvent B into A over 15 min, at 1 mL/min flow rate, with UV detection at 220 nm. Semipreparative HPLC was done with a 10 \times 250 mm, 10 μ m, C18 column (Phenomenex) with a Shimadzu LC-8A system. Solvents A and B were 0.1% TFA (v/v) in H₂O and CH₃CN, respectively. Elution was performed with linear gradients of solvent B into A over 30 min, at 5 mL/min flow rate, with UV detection at 220 nm. Fractions of adequate purity (> 95%) by analytical HPLC were pooled and lyophilized. Mass spectra were obtained by electrospray ionization with Esquire 6000 (Bruker), LTQ-Orbitrap XL (Thermo Fisher, Waltham, MA), Q-Star Pulsar (Applied Biosystems, Carlsbad, CA) or LC/MSD-TOF (Agilent Technologies) instruments, or by MALDI-TOF in a Voyager DE-STR (Applied Biosystems). Compounds **1**, **6** and **7** were synthesized as described before.⁵ On the other hand, compounds **2-3** and **8-9** were prepared according to the methodology widely used in our group⁵ (details are included in the Supplementary Information).

General Synthetic Protocol for Dendropeptide Intermediates: To a solution of 0.5 μ mol (1 equiv.) of maleimide dendron (0.24 mg of **6** or 0.5 mg of **7**) in CH₃CN (400 μ L), a solution of 1.5 equiv./maleimide group of peptide **8** or MAP **9** in 100 mM phosphate buffer, pH 7.4 (800 μ L), was added. To monitor the reaction progress, aliquots of the mixture were taken for analytical HPLC and MALDI-TOF MS analysis, and when no further changes in the HPLC profile were observed (reaction times range from few minutes to 24 h, depending on the steric hindrance), the reaction was stopped by addition of glacial AcOH. The product was then isolated by preparative RP-HPLC. The target compounds (**10-13**) were obtained in homogeneous form (> 95%) by analytical HPLC and were satisfactorily characterized by MS (see Table S1, Supporting Information).

General Protocol for Glycodendropeptide Synthesis: 0.26 μ mol of glycodendron **1** and 0.13 μ mol of peptide **2**, MAP **3** or dendropeptide intermediates **10-13** were dissolved in 100 μ L of 3:2 (v/v) THF/phosphate buffer (100 mM, pH 7.4). In a separate vial, CuSO₄·5H₂O (0.13 μ mol) was dissolved in H₂O (15 μ L) and mixed with a solution of TBTA (0.26 μ mol) in THF (33 μ L). This solution was next added to the original mixture followed by 52 μ L of sodium ascorbate (0.52 μ mol) in H₂O. After stirring for about 30 min at room temperature (the progression of the reaction was monitored by analytical RP-HPLC), the reaction mixture was lyophilized, then purified by semipreparative RP-HPLC to give target compounds **4-5** and **14-17** (see Table S1, Supporting Information).

Generation of Monocyte-Derived DCs: Fresh peripheral blood mononuclear cells (PBMCs) obtained from 40 mL of human blood from 10 healthy donors were used for monocyte purification by means of anti-CD14 microbeads following the manufacturer's protocol (Miltenyi Biotec, Germany). The CD14⁺ fraction was placed in 10% dimethyl sulfoxide and frozen for a later lymphocyte proliferation test. To generate DCs, CD14⁺ monocyte cells were incubated in complete medium (CM) containing RPMI 1640 medium (Life Technologies, Invitrogen, USA) supplemented with 10% fetal calf serum (FCS; Life Technologies, USA), streptomycin (100 μ g/mL), gentamicin (1.25 U/mL) as well as recombinant human rhGM-CSF (200 ng/mL) and rhIL-4 (100 ng/mL) (both from R&D Systems Inc., USA) for 5 days at 37 $^{\circ}$ C and 5% CO₂. The resulting immature DCs (imDC) were then recovered and used in the experiments.

DC Maturation: imDCs derived from monocytes were incubated in complete medium at 10⁵ cells/mL in 96-well plates

(Nunc AS, Roskilde, Denmark) with different GDP concentrations (10^{-3} , 10^{-4} and 10^{-5} mM). LPS at 1 mg/mL (Sigma) was used as positive control and culture media as negative control. After 72 h stimulation at 37°C in 5% CO₂, DCs were harvested and their maturation state was assessed by upregulation of CD80, CD86, and CD83 costimulatory molecules and HLA-DR, using fluorescently labelled monoclonal antibodies (BD Pharmingen, San Diego, California) in a FACSCanto II cytometer (BD Biosciences, Milpitas, California). Data were processed with FlowJo (TreeStar, Inc, Switzeland). Results were expressed as Maturation index calculated as the ratio between the percentages of stimulated DCs compared with non-stimulated DCs.

Lymphocyte transformation test (LTTs) by CFSE dilution-proliferation. For this assay, imDCs were used as antigen-presenting cells; the CD14 fraction, which included autologous lymphocytes; and GDPs at 10^{-3} , 10^{-4} and 10^{-5} mM concentration. Autologous lymphocytes at 0.5 to 1 10^7 /mL were labeled with 5,6-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE) following manufacturer's instructions. A total of 100 μ L of CFSE-labeled lymphocytes at 1.5 10^6 /mL were cultured in a 10:1 ratio with imDCs at a final volume of 250 μ L of complete medium, in 96-well plates in triplicate, with or without stimulus, for 6 days at 37°C and 5% CO₂. PHA at 10 μ g/mL (Sigma) was used as positive proliferative controls and culture media as negative control. The proliferation of different lymphocyte subsets (T cells and B cells) were assessed by flow cytometry, analysing the percentage of CD3, CD4, CD8 and CD19 cells that show a dim expression of CFSE (CFSE^{dim}). The results were expressed as proliferation index (PI), calculated for each subset as ([% CFSE^{dim} stimulated lymphocytes-lymphocyte-DC]/ % CFSE^{dim} unstimulated lymphocytes).

Ethics Statement. The institutional review board "Ethic Committee of Málaga Hospital" approved the study. These studies were carried out in accordance with the Declaration of Helsinki. Oral and written informed consent for all the diagnostic procedures was obtained from subjects before included in the study.

Statistical Analysis. The maturation and proliferation results after culturing with the different GDPs were compared with nonparametric analysis for related samples (Wilcoxon test). All reported *P* values represent 2-sided tests, with the level of significance set at the probabilities of *p* < 0.05.

Supporting Information: Mass spectra, HPLC chromatograms and toxicity assays.

Conclusions

Our studies indicate that viral peptides such as NP₃₆₆₋₃₇₄ displayed in the novel GDPs are recognized by the immunological system inducing DCs maturation and therefore an increase in the T lymphocyte proliferation. We have found that this effect is oriented to a higher proliferation for CD8 T cells, the typical cytotoxic cells in an immunological response against infectious diseases. These responses are directly related to peptide valency within the structure.

Moreover, we have found that the activation marker CD83 appears to act as a maturational DC marker directly related to the proliferative response of CD8 T cells. These promising results suggest that this marker could be useful in evaluating candidates suitable to be used as viral vaccines inducing a T cell cytotoxic response. Further studies are needed to better

characterize the immunological response induced by these structures.

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A GDP displaying the NP₃₆₆₋₃₇₄ influenza A virus epitope induces an immunostimulatory effect, emerging as a potential flu vaccine.

