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Article

Impact of N-Terminal PEGylation on Synthesis and Purification of Peptide-Based Cancer Epitopes for Pancreatic Ductal Adenocarcinoma (PDAC)

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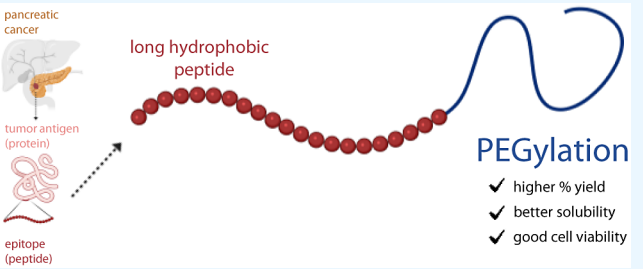
ABSTRACT: Peptide-based cancer vaccines have shown promising results in preclinical trials focusing on tumor immunotherapy. However, the presence of hydrophobic amino acid segments within these peptide sequences poses challenges in their synthesis, purification, and solubility, thereby hindering their potential use as cancer vaccines. In this study, we successfully synthesized peptide sequences derived from mesothelin (MSLN), a tumor-associated antigen overexpressed in pancreatic ductal adenocarcinoma (PDAC) by conjugating them with monodisperse polyethylene glycol (PEG). By PEGylating mesothelin epitopes of varying lengths (ranging from 9 to 38 amino acids) and hydrophobicity (60−90%), we achieved an effective method to improve the peptide yield and facilitate the processes of synthesis and purification. PEGylation significantly enhanced the solubility, facilitating the single-step purification of lengthy hydrophobic peptides. Most importantly, PEGylation did not compromise cell viability and had little to no effect on the immunogenicity of the peptides. In contrast, the addition of a palmitoyl group to increase immunogenicity led to reduced yield and solubility. Overall, PEGylation proves to be an effective technique for enhancing the solubility and broadening the range of utility of diverse long hydrophobic peptides.



INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive forms of cancer, killing about nine in ten patients within 5 years after diagnosis.[1,2] PDAC is extremely difficult to diagnose at its early stage,[3] and most patients are diagnosed only after the cancer is metastasized.[4] Currently, the only curative therapy for PDAC is surgical resection, but fewer than 20% of patients have resectable tumors at the time of diagnosis.[2] More than 80% of patients are diagnosed with advanced-stage tumors, for which median survival with chemotherapy is less than 1 year.[4,5] In stark contrast to other tumor types, targeted therapies in multiple large-scale trials have been unsuccessful for PDAC.[6−8] Nonetheless, a broader understanding of the resistant PDAC tumor and its intricate interactions with the immune system has opened new avenues of treatment.

Recently, the incorporation of immunotherapy in the treatment of various solid tumors has marked a paradigm shift in oncology.[9,10] Immune-based therapies aim to recruit and activate immune cells to eliminate tumor cells. Among these therapies are peptide-based cancer vaccines.[11] Cancer vaccines work by the same principles as vaccines for other diseases, where the active ingredient triggers an immune response that generates a long-term immunity to a foreign antigen. Cancer vaccines train the immune system to identify tumor antigens as "foreign," targeting and eliminating cancer cells.[12,13] Peptide-based cancer vaccines are made up of a sequence of amino acids derived from tumor antigens that are either present in both normal and cancer cells but overexpressed in the latter (tumor-associated antigen) or solely found in cancer cells (tumor-specific antigen).[14] From these tumor antigens, the selected sequence can be in the form of a single epitope, a long peptide chain containing multiple epitopes or a cocktail containing multiple separate epitopes.[15] Long multi-epitope peptide vaccines are ideal since they elicit a strong immune response and are less prone to enzymatic digestion and elimination from the body (Figure 1).[16] However, the synthesis of combined long peptides comes with challenges in handling and solubility.[17]

Since its introduction in 1963 by Merrifield,[18] solid-phase peptide synthesis (SPPS) has become the strategy of choice for the synthesis of peptides used in research and for the vast

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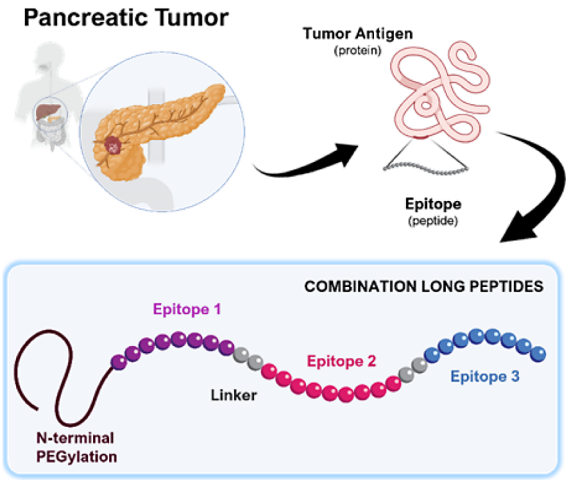

Figure 1. PEGylated peptide-based cancer vaccines for PDAC immunotherapy.

majority of peptide-based pharmaceutical ingredients.[19,20] Synthesis of long peptides (>25 amino acid (aa) residues) implies a greater likelihood of being accompanied by many impurities derived from incomplete amino acid couplings[21] and secondary reactions in the deprotection,[22] coupling,[23] or cleavage steps.[24] There are also small and medium-sized peptides (up to 10 and 25 aa residues, respectively), which are very difficult to synthesize with a decent quality by SPPS.[25] The impurities, mostly truncated peptides, are often difficult to chromatographically resolve from one another, especially as the sequence grows longer. This makes the mandatory purification step much more difficult.[26] Furthermore, the recovery of pure products declines with increasing hydrophobicity of the sequence.[27] Finally, the hydrophobicity of certain sequences greatly limits their downstream application, as they are often restricted to dimethyl sulfoxide (DMSO) for solubilization, which then needs to be diluted with aqueous buffers for compatibility in bioassays due to the toxicity of most organic solvents.[28]

We hypothesized that these synthesis, purification, and solubility problems can be overcome by conjugating polyethylene glycol (PEG) to the hydrophobic peptide sequences. Herein, we delineate our efforts toward this end using combined peptide sequences covering epitopes from well-described tumor-associated antigens overexpressed in PDAC.

RESULTS AND DISCUSSION

In this study, we selected peptide sequences that cover mesothelin epitopes that are known or predicted to elicit an immune response against PDAC. These selected mesothelin epitopes were synthesized either as a single epitope (short) or as a multiple epitope (long) peptide sequence. A palmitoyl group was also added to increase the immunogenicity of the sequences. We investigated the impact of PEGylation on the synthesis, purification, and solubility of the peptides. Furthermore, we conducted bioassays to evaluate the effect of PEGylation on the cell viability and immunogenicity of the peptides.

Design of Peptide Sequences. Mesothelin (MSLN) is a 40 kDa cell surface glycoprotein demonstrated previously by gene expression analysis to be a tumor marker for PDAC.[29,30] It has limited expression in normal tissues and has significant overexpression in almost 90% of PDAC cases,[31] making it an attractive candidate for cancer immunotherapy. MSLN overexpression has been demonstrated to promote proliferation, advancement of the cell cycle, and survival of cancer cells.[32,33] In many PDAC cases, it is also associated with shorter overall survival and resistance to chemotherapy, which underscores the importance of targeting MSLN.[34]

As such, we selected three hydrophobic MSLN epitopes, MSLN 1−3 that have been predicted to trigger an immune response using publicly available epitope prediction servers,[35,36] namely SYFPEITHI[37] and BIMAS.[38] The sequences were joined together using a cathepsin-like cleavage site (KK or Lys-Lys)[39] to obtain the long-combined peptide MSLN 4 (Table 1). The peptide sequences MSLN 1−4 include a range of lengths, covering amino acid residues from 9 to 38, and exhibit diversity in relative hydrophobicity levels, extending from 60% to 90%. The peptides were PEGylated to enhance their solubility and palmitoylated to increase their immunogenicity.[40,41] Although the specific mechanisms for how fatty acid increases immunogenicity are still being discussed, the positive effect has been reported.[40] Modifications such as those in peptide sequences derived from self-antigens are necessary given their low immunogenicity, potential for inducing immune tolerance, and the generally poor immune response against tumor antigens.[42]

Peptide PEGylation. *Synthesis of Fmoc-PEG 23 Propionic Acid 4.* For peptide PEGylation, we first synthesized a monodisperse heterobifunctional PEG with Fmoc and a carboxylic acid functional group. It was crucial to start with high-purity polyethylene glycol for the PEGylation of peptides to reduce the necessary purification work later and to ensure a single molecular identity. We chose a >1000 Da PEG since it is one of the very few long PEG oligomers that are available in monodisperse form in multigram quantity.

Monodisperse Fmoc-PEG 23 propionic acid was synthesized from monodisperse amino PEG acid **1** obtained from Polypure (see Scheme S1). Amino-PEG acid was first converted to amino-PEG methyl ester **2** by a repeated cycle of heating in methanol with HCl and evaporation under reduced pressure. Amino-PEG methyl ester **2** was protected with a Fmoc group

Table 1. Mesothelin Epitopes for PDAC Cancer Vaccines

epitope	coverage in MSLN	sequence	length	% hydrophobic residues[a]	hydrophilicity score[b]
MSLN 1	541−555	PLTVAEVQKLLGPHV	15	60	−0.34
MSLN 2	117−126	ALPLDLLLFL	10	90	−1.08
MSLN 3	20−28	SLLFLLFSL	9	78	−1.49
MSLN 4	541−555,117−126,20−28	PLTVAEVQKLLGPHVKKALPLDLLLFLKKSLLFLLFSL	38	66	−0.46

[a]Peptide relative hydrophobicity (% hydrophobic residues) was calculated using the peptide2.com hydrophobicity calculator. [b]Hydrophilicity scores were calculated via the BACHEM peptide calculator tool (https://www.bachem.com/knowledge-center/peptide-calculator/).

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giving compound **3**, and the ester functionality was cleaved upon treatment with HCl (aq) to give acid **4**. Fmoc-PEG 23 propionic acid **4** (yellowish oil) was recovered by extraction with dichloromethane (DCM) in a yield of 94% (30 g) with a purity of >98% based on LC−MS analysis. The ^{1}H and ^{13}C NMR analyses further confirmed the purity of the compound. The total ion chromatogram (retention time: 7.5 min) and mass spectrum of the final product (ESI-MS: $[M + H]^+$ = 1368.7) are shown in Figure 2. The ^{1}H and ^{13}C NMR spectra are shown in Figures S1 and S2.

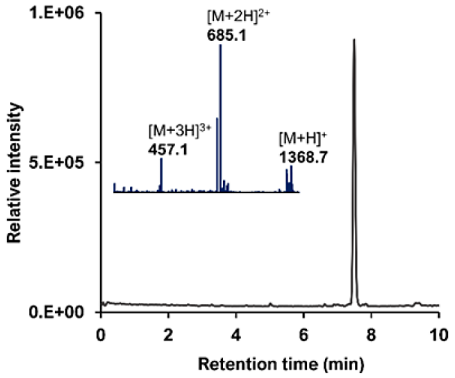

Figure 2. HPLC chromatogram and MS spectrum (inset) of Fmoc-PEG 23 propionic acid **4**. Retention time at 7.5 min in a 5 to 75% B gradient (A: 0.1% TFA in H_2O and B: CH_3CN) for 10 min. ESI-MS: $[M + H]^+$ = 1368.7, $[M + 2H]^{2+}$ = 685.1, $[M + 3H]^{3+}$ = 457.1.

Ensuring a high PEG oligomer purity is of utmost importance, as even a one-unit deviation in PEG length (either $n - 1$ or $n + 1$) can persist throughout the subsequent peptide synthesis. This variance in the oligomer length significantly increases the potential impurities during peptide purification. Purifying peptides with PEG impurities presents a greater challenge since a PEG unit weighs 44 Da, whereas the smallest amino acid residue, glycine, weighs 57 Da.

The successful synthesis of monodisperse Fmoc-PEG 23 propionic acid **4** was reported by Wang et al.,[41] where they used a macrocyclic sulfate-based approach starting from PEG 4 diol on a 5 g scale. However, an assessment of the purity of the resulting product was not discussed. Herein, we have synthesized a monodisperse Fmoc-PEG 23 and evaluated the oligomer purity of the amino-PEG acid using LC−MS in selected ion mode (SIM) looking for $n - 2$, $n - 1$, and $n + 1$ impurities. A linear range in which the injection volume is proportional to the concentrations (area under the curve) of the different PEG oligomers was identified (Figure 3). Our results showed an average purity of 99.5% across varying injection volumes (Table 2).

Synthesis of PEGylated and Palmitoylated Peptides. The peptides were synthesized manually or automatically using a Liberty Blue automated microwave peptide synthesizer following a standard Fmoc/*t*-Bu-based solid-phase synthesis protocol (SPPS).[43] The bulk of these sequences was synthesized by microwave-assisted automated synthesis, as it provides quick, reliable, and reproducible production of crude peptides. Furthermore, our previous experiences with manual synthesis of these types of peptides have pointed to the need for automated synthesis for MSLN 3 and MSLN 4 as these are the most synthetically challenging of the four peptides. The exception here is MSLN 2 as **P13** and **P14** are not quite as

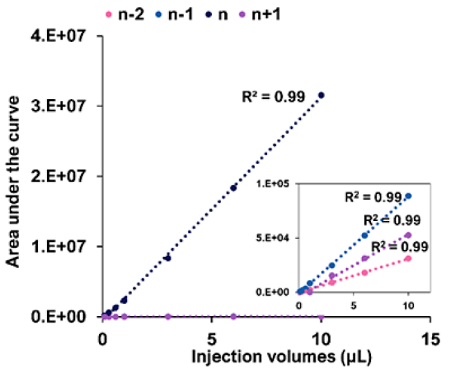

Figure 3. Analysis of amino-PEG 23 propionic acid oligomer purity. Linearity of different injection volumes (0.1, 0.3, 0.6, 1.0, 3.0, 6.0, and 10.0 μL) and PEG oligomer concentrations (area under the curve of chromatogram).

Table 2. Percent Purity of the PEG Oligomers[a]

	oligomer percent %			
injection volume (μL)	$n - 2$	$n - 1$	n	$n + 1$
0.1	0.00	0.35	**99.65**	0.00
0.3	0.13	0.29	**99.57**	0.00
0.6	0.12	0.26	**99.62**	0.00
1	0.09	0.35	**99.56**	0.00
3	0.11	0.30	**99.42**	0.18
6	0.10	0.28	**99.45**	0.17
10	0.10	0.28	**99.46**	0.17

[a]The selected ion monitoring (SIM) method in HPLC−MS was used to quantitate the oligomer purity of different injection volumes for 0.4 μM concentration of amino-PEG acid.

challenging and could be produced by manual synthesis. Fmoc Rink Amide AM resin was used as solid support, and the first amino acid was coupled to obtain a 0.5 mmol/g loading. The remaining free sites were capped by acetylation, followed by deprotection using 20% piperidine/dimethylformamide (DMF). Further residues were incorporated using standard protected Fmoc amino acids, *N*,*N*′-diisopropylcarbodiimide (DIC), and OxymaPure in DMF as coupling reagents. PEGylation and palmitoylation were prepared manually using monodisperse Fmoc-PEG 23 propionic acid **4** and palmitic acid (Scheme S2). Subsequent cleavage was performed by treatment with TFA/TIS/H_2O (95/2.5/2.5). The cleavage mixture was precipitated with cold diethyl ether and centrifuged, and the resulting pellet was redissolved in H_2O/ACN (1:1) for lyophilization, followed by characterization by HPLC and LC−MS.

The crude yield and recovery of the synthesized peptides are summarized in Table 3. The quantity (milligrams) of crude peptides nearly doubled upon PEGylation, as expected. The incorporation of the high molecular weight PEG moiety (1367 Da) naturally increases the amount of crude oil while maintaining the integrity of the peptide sequence. This offers a distinct advantage as it provides a larger amount of material available for subsequent purification and use in bioassays.

Crude Peptides. Crude amounts (mmol) of PEGylated products **P2**, **P8**, and **P11** for MSLN 1, 3, and 4 epitopes are higher than the unmodified peptides **P1**, **P7**, and **P10** and significantly higher than their palmitoylated versions (**P3**, **P9**, and **P12**), respectively. This trend is not seen for MSLN 2, where the highest recovery is observed for the unmodified

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Table 3. Summary of Yield, Purity, and Solubility of Synthesized Peptides

epitope	ID	N-terminal	MW (Da)	mmol obtained[a]	% purity	% yield[b]	mmol purified	% purity	% recovery[c]	solubility (1 mg/mL)
MSLN 1	**P1**	NH_2−	1599.9	0.080	84	67	0.025	>99	37	water
	P2	PEG23−NH−	2728.3	0.084	81	68	0.016	>99	24	water
	P3	Palm−NH−	1838.4	0.046	83	38	0.009	95	22	ACN/water (1:1)
MSLN 2	**P4**	NH_2−	1126.5	0.095	82	78	0.048	99	61	ACN/water (1:1)
	P5	PEG23−NH−	2254.8	0.074	86	63	0.046	96	70	water
	P6	Palm−NH−	1364.9	0.072	76	55	0.012	99	23	DMSO
MSLN 3	**P7**	NH_2−	1051.3	0.059	56	33	0.004	95	11	ACN/water (1:1)
	P8	PEG23−NH−	2179.7	0.072	55	40	0.014	95	33	water
	P9	Palm−NH−	1289.8	0.036	75	27	0.013	98	47	DMSO
MSLN 4	**P10**	NH_2−	4256.4	0.058	71	41	0.011	73[d]	19	ACN/water (1:1)[d]
	P11	PEG23−NH−	5384.7	0.069	81	56	0.020	>99	36	ACN/water (1:1)
	P12	Palm−NH−	4494.8	0.049	57	28	0.015	97	53	ACN/water (1:1)

[a]The peptides were synthesized in a 0.1 mmol scale. [b]% Yield = (crude mmol × crude purity)/(theoretical mmol). [c]% Recovery = (purified mmol × purity)/(crude mmol × crude purity) × 100. [d]Cannot be purified to more than 90% due to a des-Leu impurity. Gelates in water/acetonitrile at high concentrations.

peptide **P4** while the palmitoylated **P6** and PEGylated **P5** modified peptides display similar levels of recovery. Since the automated peptide synthesis process for the same base sequence is the same up to the final modification of the N-terminus, the variation in yield of the peptide must be related to the efficiency of the cleavage from the solid support and the following workup. This can be attributed to better precipitation in the cold diethyl ether. Palmitoylated peptides do not readily precipitate unless TFA is removed by evaporation first, a requirement not common for most cleaved synthetic peptides. The palmitoyl moiety increased the hydrophobicity of the sequence and made it more difficult for these peptides to adequately precipitate in diethyl ether when TFA is present. Moreover, it is worth noting that the sequences exhibit a tendency toward greater hydrophobicity, as indicated by their negative hydrophilicity scores. Specifically, the base sequences of MSLN 2, MSLN 3, and MSLN 4 are predominantly composed of hydrophobic amino acid residues (Table 1). This makes precipitation challenging even in the absence of fatty acids, as we observed *in situ*. This is a hallmark of hydrophobic peptides, whereas most peptides carry enough polar and charged residues to not be solvated by diethyl ether and thus precipitate. Unlike these two cases, the PEGylated peptides that we studied precipitated easily and without the need to fully remove TFA first.

When calculating yields, which consider the purity of the crude product, the following trends appear: first, all palmitoylated peptides **P3**, **P6**, **P9**, and **P12** have very low yields compared with their counterparts. Second, yields for PEGylated peptides are either higher (**P8** and **P11**) or similar (**P2**) compared to the unmodified peptides. The exception is, again, MSLN 2, which is the only case where the unmodified peptide **P4** shows a better yield than the PEGylated peptide **P5**. The low mmol values obtained inherently affect the yield, this is especially noticeable for the palmitoylated peptides. Individually, for example, MSLN 1 variants all have similar purities, and so the low absolute amount of mmol of crude obtained peptide **P3** makes it the product with the lowest yield. With a trend very clear for palmitoyl peptides, we compared the situation between the unmodified peptide and the PEGylated peptide. The driving force involved between all these pairs of peptides is similar. MSLN 2 seems to be a particular case. It can be proposed that the single positive charge provided by TFA in the N-terminal free amine is overall more effective in the smaller unmodified peptide **P4** than in the larger PEGylated peptide **P5** and thus precipitates better in diethyl ether, which explains the excellent absolute recovery. This in turn drives the high yield of the unmodified peptide compared to that of the PEGylated peptide **P5** and certainly with respect to that of the palmitoylated peptide **P6**.

Two other versions of MSLN 2 were synthesized in which we attached both PEG and palmitoyl to the base sequence. These versions are **P13** (Palm-PEG11-MSLN 2) with a shorter PEG 11 moiety and **P14** (Palm-PEG23-MSLN 2) with a longer PEG 23. Here, our findings revealed that both the length of the PEG chain and the hydrophobicity of the underlying amino acid sequence affect the precipitation of the peptides. Contrary to **P14**, which had a longer PEG chain and rapidly precipitated, **P13** with the shorter PEG chain did not undergo precipitation in a single step. This suggests that throughout the precipitation and subsequent washing stages after cleavage a certain amount of peptide is inadvertently lost due to incomplete precipitation. PEGylation using a longer PEG chain mitigates this problem, leading to higher yields.

Purified Products. The final mmol obtained of purified products (as shown in Table 3, column 8) was higher for the PEGylated peptides **P8** and **P11** in comparison to their unmodified counterparts **P7** and **P10**, respectively, although it should be noted that MSLN 2 escapes this trend. The other exception is MSLN 1, in which the unmodified peptide **P1** was obtained in a larger amount than that of the PEGylated peptide **P2**. The absolute recovery in mmol of pure PEGylated peptides increased in all cases when compared to their palmitoylated versions and even the least hydrophobic sequence, namely MSLN 1, saw a substantial increase in recovered material. For MSLN 3, however, this recovery is not significantly greater. Palmitoylated MSLN4 peptide **P12** shows better rates of recovery compared to their counterparts **P10** and **P11**. This is due to the distribution of impurities (see the Supporting Information) further away from the main peptide peak. While the overall purity is lower than that for **P10** and **P11**, this distribution facilitates the purification. This is not the case for **P10** and **P11**, in which we observe most of the impurities overlapping the main peak. This leads to sacrificing more of the desired product as perfect resolution of impurities close to the product peak is not possible. Obtaining a larger quantity of pure product from a single synthetic and purification process is the most important advantage that

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PEGylation can bring to peptide production, in addition to the well-established benefits it offers for enhancing bioavailability.

In terms of percent recovery, which considers the recovery of a product with an acceptable purity of 95% or greater from the initially available peptide from the crude mass (refer to Table 3, column 10), the most noticeable effect can be seen with MSLN 2, in which recovery improves substantially for PEGylated peptide **P5**, particularly when compared to that of the palmitoylated sequence **P6**. While the initial specific yield was highest for **P6** (mainly due to a +96 Da impurity, likely trifluoroacetylation, appearing sometimes but mostly in sequences that do not carry a palmitoyl moiety and thus capped N-terminal amine), recovery of this peptide was extremely low, unlike the case for **P5**. Recovery of palmitoylated peptides is, however, higher for MSLN 3 and 4. In the case of MSLN 3, this is mainly due to the higher purity of the **P9** crude, while for MSLN 4, the impurities of **P12** are spread widely across the gradient and are thus more easily resolved.

For MSLN 3, the trend seen for MSLN 2 is also true with respect to the base sequence in peptide **P7**, if not for the palmitoylated version **P9**, as recovery in the relative percentage is better. Nevertheless, both **P8** and **P9** have better absolute recoveries than base sequence **P7** (0.004 mmol **P7**, 0.014 mmol **P8**, and 0.013 mmol **P9**). The latter pattern is seen also in MSLN 4, and it is more noticeable compared to the base sequence in peptide **P10**. In fact, in this case, a des-Leu impurity (among others), present in the long MSLN 4 sequence, could not even be resolved, and we were unable to obtain a purified peptide **P10** (at best, we improved the purity from 71 to 73%; see the Supporting Information). This is likely due to three issues: first, PEGylated peptides generate sharper chromatographic peaks, whereas both the palmitoylated and unmodified versions have more triangular and wider dragging peaks (see the Supporting Information for chromatograms). This affects resolution, especially in semipreparative scales, which means that collecting less of the peak is necessary to maintain adequate purities, which causes the loss of more product in exchange for greater purity. For MSLN 4, the increased retention in the column of the unmodified sequence **P10** giving these "dragging" peaks is less affected by PEG23, yet PEGylation proves adequate for obtaining pure product **P11**.

The second variable is the hydrophobicity. The PEG moiety has a greater effect on the solubility of MSLN 2 peptide **P4** than for the more hydrophilic sequence MSLN 1 **P1**, in which the effect is even reversed (**P2** has a marginally higher retention time in a C18 stationary phase). Hydrophobic sequences are more retained in the column due to stronger interactions with the hydrocarbon chain of the stationary phase, whereas the hydrophilic PEG moiety favors solvation in the mobile phase. This is also seen in MSLN 3 comparing unmodified peptide **P7** and PEGylated version **P8**.

The third variable is the size. The shorter MSLN 2 peptide **P4** is more affected by PEGylation as seen for peptide **P5**, and the effect on its recovery is greatly increased compared to a longer sequence like MSLN 4 (**P10** and **P12**), in which the effect is lessened.

In a few cases, however, we observed a better recovery rate in palmitoylated peptides. Although due to the way this parameter was calculated, this was mainly an effect of the crude simply being purer to begin with or containing impurities that were easier to resolve chromatographically. This is something to be noted, as besides the usual synthetic issues, especially with deletions in longer sequences, the potential modification by secondary reactions in downstream processing, namely, during cleavage, is yet another source of problems. Mass spectrometry analyses of the studied sequences showed an unwanted +96 Da modification due to trifluoroacetylation of (likely) serine and threonine residues which was more prevalent when the N-terminal amine was not capped. It must also be noted that palmitoylation is not a random modification that we chose to test. It was included in the sequence to improve its immunogenic potential. As such, we must focus mainly on what happens between PEGylation and palmitoylation and the possibility of combining the two. The potential arises from the fact that PEGylation offers a suitable modification strategy for enhancing the solubility of a hydrophobic peptide as well as other benefits upon application in biological systems such as increasing its half-life. PEGylation can be implemented prior to introducing a palmitoyl moiety with the aim of enhancing solubility. In turn, this should lead to improvements in precipitation in diethyl ether, chromatographic resolution, and the reduction of interactions with the stationary phase of semipreparative chromatographic columns. These collective enhancements would contribute to obtaining a greater yield of a pure product. It is anticipated that introducing a PEG 23 moiety prior to palmitoylation would combine the initial high purity observed in some palmitoylated products with the improved crude yields seen in the PEGylated variants as well as their generally higher absolute recoveries. This approach would effectively counteract the significant hydrophobic nature of palmitoylated products and address the tendency toward cleavage-specific secondary reactions similar to the likely *O*-trifluoroacetylation of serine/threonine that we observed in peptides with an unmodified N-terminal amine.[44]

Solubility. Visual assessment of the solubility at 1 mg/mL in some common solvents for peptides was carried out. PEGylation increased the solubility of the hydrophobic base sequences in water and facilitated biological testing. Palmitoylation, however, decreased the solubility in water, as expected for a fatty acid chain attachment.

PEGylation increased the solubility of base sequences **P4** and **P7** from ACN/water (1:1) to 100% water (Table 3). Palmitoylation made peptides **P6** and **P9** insoluble in ACN/water (1:1) and soluble only in DMSO. No significant changes in solubility were observed for PEGylation of **P1** to **P2**, while a decrease of solubility in water is observed with palmitoylation, **P3**.

To test the increase in the solubility of palmitoylated peptides, we performed a preliminary study in which we synthesized one batch of the base sequence of MSLN 2 and split it to make three modifications (Figure 4): (A) palmitoylation **P6** and PEGylation with either, (B) PEG 11 **P13**, or (C) PEG 23 **P14**, followed by palmitoylation. We observed decreasing retention times (Figure 4A–C): 9.2 min for **P6** (falling outside of the gradient range), 6.4 min for **P13**, and 5.4 min for **P14**. We also observed much sharper peaks for the two PEGylated peptides **P13** and **P14**. These results point in the direction we proposed, although it should be noted that to confirm this, careful individual analysis of yields and recoveries must also be performed.

The significance of these PEGylated epitopes being more soluble in aqueous medium than the originally designed epitopes while still retaining the properties for downstream applications cannot be overstated. PEGylation proves to be an

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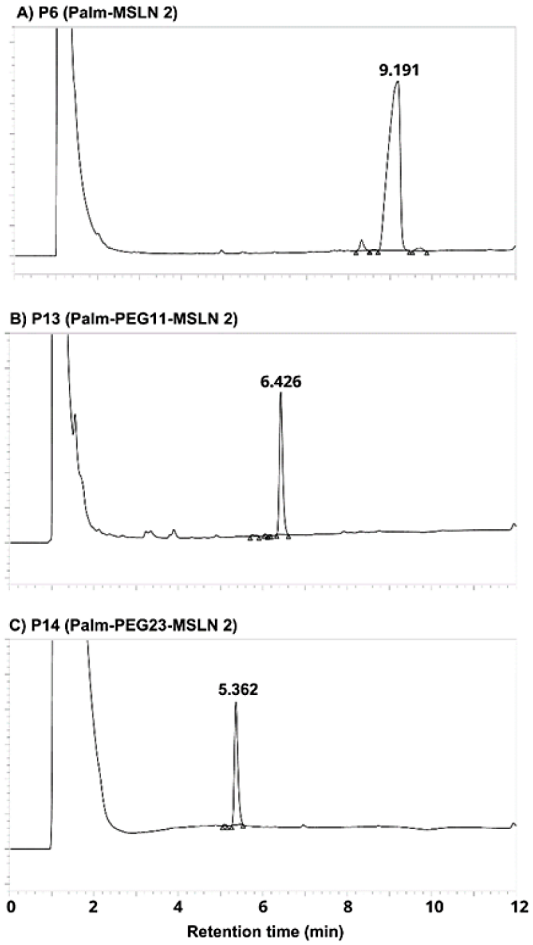

Figure 4. Chromatograms of (A) **P6** (Palm-MSLN 2), (B) **P13** (Palm-PEG11-MSLN 2), and (C) **P14** (Palm-PEG23-MSLN 2) in 60−100% A: 0.045% TFA in H_2O and B: 0.036% TFA in CH_3CN for 8 min.

effective modification technique for enhancing solubility and broadening the range of utility of bioactive substances like peptides.

Effect of PEGylation of Peptides in Cell Stimulation. To verify whether the PEGylation of the peptides would influence the proliferation and activation of immune cells, we tested splenocytes from 15 C57BL6 male mice immunized with PLGA nanoparticles containing the well-known adjuvants, poly I:C and R848.[45,46] The cells were restimulated in vitro with positive control (Concanavalin A) or the unmodified peptides **P1**, **P4**, and **P7**, PEGylated peptides **P2**, **P5**, **P8**, and **P11**, and palmitoylated long peptide **P12** soluble in ACN/water (1:1) at 10 μg/mL for 48 h.

After the stimulation, a viability assay was performed, and we found no difference in the proliferation of the cells when stimulated with the PEGylated peptides, in comparison with the unmodified version (Figure 5A). For **P12**, the cells were more proliferative. This suggests that palmitoylation of MSLN 4 is not toxic and induces cell growth.

The activation of the stimulated cells was assessed using ELISA by measuring interferon-gamma (IFN-γ) levels. IFN-γ is a marker for the activation of cytotoxic T $CD8^+$ cells mediated by Th1 cells and is related to antiproliferative, pro-apoptotic, and antitumoral activity as well as tumor immune surveillance. As such, its secretion is expected to increase in antiviral and antitumoral immune responses. This cytokine is positively associated with cancer survival. It is thus a useful marker to study whether the peptides are activating antitumoral T $CD8^+$ cells.[47]

We did not find a significant difference in the levels of IFN-γ secreted in the supernatant of the cells when stimulated with PEGylated peptides compared to cells stimulated with unmodified peptides except for MSLN 2 **P5** (Figure 5B). This difference can be attributed to the possibility that the long PEG chain might have hindered the interaction of the active site of the short MSLN 2.

The increased levels of IFN-γ for both the palmitoylated **P12** and PEGylated long peptide **P11** (modified MSLN 4) demonstrate that indeed, multiple-epitope long peptide sequences can elicit a stronger immune response than single-epitope short peptide sequences. The unmodified long peptide **P10** was not tested since the peptide could not be purified to more than 95% purity.

In this case, PEGylation proves to be an effective technique for enhancing the solubility and broadening the range of utility of diverse long hydrophobic peptides. This result also further enhances our proposed strategy of combining both modifications in the MSLN 4 sequence.

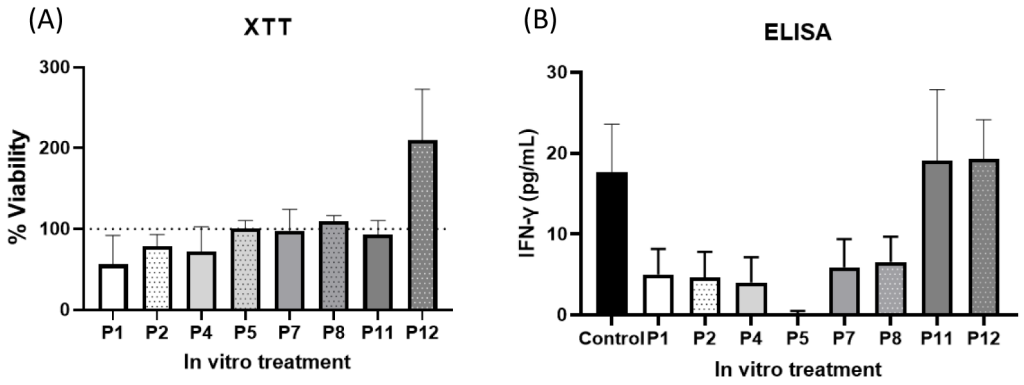

Figure 5. Effect of cell stimulation with PEGylated peptides. Splenocytes from vaccinated mice (adjuvants in PLGA nanoparticle vaccines) were stimulated with unmodified and PEGylated peptides for 48 h. (A) PEGylation of the peptides did not have influence on the viability of the cells. Data was normalized to the absorbance levels of nonstimulated cells. Cell proliferation was measured by the XTT assay. (B) PEGylation of the peptides did not change the levels of IFN-γ in the supernatant measured by ELISA except for MSLN 2.

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CONCLUSIONS

In this study, we synthesized peptide sequences from mesothelin, a tumor antigen overexpressed in PDAC and conjugated them with monodisperse PEG. N-terminal PEGylation of peptides of varying lengths (<40 aa residues) and hydrophobicity (up to 90% of hydrophobic residue content) proved to be an excellent method to increase yield and recovery during synthesis and purification. PEGylation also improved solubility, making it possible for a long multiepitope hydrophobic peptide to be purified in one step and biologically tested. Cell viability and immunogenicity of peptides were not significantly affected by PEGylation. Peptides exceeding 40 amino acids with a hydrophobic residue content of more than 80% are promising candidates for PEGylation with extended PEG units such as PEG-45 or longer. In contrast, our results also underscore the influence of palmitoylation on peptide synthesis and purification. This is also in line with the trend of producing longer synthetic peptides as bioactive compounds. The addition of a palmitoyl group, aimed at increasing immunogenicity, led to lower yield and solubility, thereby complicating subsequent biological evaluation. The effects of palmitoylation on synthesis and purification are sequence dependent. However, we observed a general trend toward more difficult processing of the peptide. This is very clear in the cases of MSLN 2 and MSLN 3, where elution from a C18 stationary phase column was not quantitative (data not shown) and even a C8 stationary phase, required aggressive gradients of 80−100% to 90−100% of organic mobile phase, a drastic change from their unmodified or PEGylated variants (see the Supporting Information). Purifying by semipreparative chromatography, a similar trend can be observed for MSLN 4 although it is less pronounced. In addition, the MSLN 4 base sequence can not be purified in one step (Table 3), a problem that was solved by PEGylation. A combined strategy of palmitoylation and PEGylation, especially for longer sequences with inherent synthetic difficulties, such as MLSN 4, is therefore a sound approach to attempt in future assays, as it represents a compromise between improving the intended biological activity while decreasing difficulties in synthesis and purification and at the same time maintaining adequate solubility for biological evaluation.

EXPERIMENTAL PROCEDURES

General Experimental. *Reagents and Solvents.* The monodisperse PEG products (amino-PEG 23 acid and Fmoc-PEG 11 propionic acid) were provided by Polypure AS (Oslo, Norway). Solvents, dichloromethane (DCM), tetrahydrofuran (THF), and acetonitrile (ACN) used for the synthesis of the monodisperse PEG were obtained from Chiron AS (Trondheim, Norway). Potassium *tert*-butoxide (KO*t*-Bu), *tert*-butyl acrylate (*t*-Bu acrylate), mesyl chloride (MsCl), triethylamine (TEA), ammonia (NH_3), trifluoroacetic acid (TFA), and *N*-(9*H*-fluoren-9-ylmethoxycarbonyloxy) succinimide (Fmoc-ONSu) were purchased from Merck KGaA (Darmstadt, Germany).

The 20 standard Fmoc amino acids, Fmoc Rink Amide AM resin (loading 0.71 mmol/g, 100−200 mesh), and 2-cyano-enzotriazolino)acetate (OxymaPure) were purchased from Iris Biotech GmbH (Marktredwitz, Germany). Piperidine, DCM, *N*,*N*-dimethylformamide (DMF), ACN, diethyl ether (Et_2O), and dimethyl sulfoxide (DMSO) were supplied by Carlo Erba Reagents (Val-de-Reuil, France). Palmitic acid, triisopropylsilane (TIS), acetic anhydride, and *N*,*N*-diisopropylethylamine (DIPEA) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) and TFA were obtained from Fluorochem Ltd. (Hadfield, UK). *N*,*N*′-diisopropylcarbodiimide (DIC) was purchased from KEMI-LAB Organics Ltd. (Veszprém, Hungary). Formic acid was purchased from Merck KGaA (Darmstadt, Germany). Rink Amide ProTide LL resin (0.18 mmol/g loading, 100−200 mesh) was obtained from CEM Corp (Charlotte, US). Poly(vinyl alcohol) (PVA, M_w 9000−10,000 80% hydrolyzed), polyinosinis-polycytidylic acid sodium salt (Poly(I:C) or pIC), resiquimod (R848), and chitosan oligosaccharide lactate (ave. M_n = 5000) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). PLGA (M_w: 17,000, PURASORB PDLG 5002A) was obtained from Corbion PURAC (Amsterdam, NL).

Characterization. *Characterization of Monodisperse Fmoc PEG-23 Propionic Acid.* High-performance liquid chromatography−tandem mass spectrometry (HPLC-MS) was carried out by using an Agilent 1269 Infinity II LC/MSD System. The HPLC system was operated using Solutions A (MilliQ water, 0.1% TFA) and B (ACN) running through a 30 mm × 2.1 mm analytical C18 column (Avantor). Single quadrupole MS was operated with an electrospray ionization source (ESI) using the following settings: ionization mode: positive, drying gas temperature = 350 °C, capillary voltage = 3000 V, drying gas flow = 12.0 L/min, nebulizer gas pressure = 35 psig and acquisition range = 100−1000 or 3000 m/z. ^1H and ^{13}C NMR spectra were recorded at 25 °C on a Bruker Ascend 600 MHz Avance NEO spectrometer.

Characterization of Peptides. Crude products obtained from the cleavage step were characterized by HPLC to assess purity, using Alliance chromatographic equipment (Waters Corp., US) with a XBridge BEH C18 column (dimensions 4.6 mm × 100 mm, particle size 3.5 μm). A gradient of 5−100% of B in min was used where A: Milliq water with 0.045% TFA and B: ACN with 0.036% TFA). For more hydrophobic peptides, a Kinetex C8 column (dimensions 4.6 × 100 mm, particle size: 5 μm) was used with 50−100% and 80−100% B gradients, depending on the peptide. Data was acquired at 220 nm and analyzed with the Empower 3 Pro software (build 3471, Waters Corp., US). Crude peptides were also characterized by LCMS to confirm their identity. Low-resolution mass spectrometry analyses were carried out in an AllianceHT chromatograph with a micromass ZQ mass spectrometer (Water Corp., US). Data was acquired and analyzed with the MassLynx software (v4.1, Waters Corp., US).

Experimental Section. *Synthesis of Monodisperse Fmoc-PEG 23 Propionic Acid* ***4***. Amino-PEG acid **1** was converted to amino-PEG methyl ester **2** by a repeated cycle of heating in methanol with HCl and evaporation under reduced pressure. The following solutions were then prepared: (A) amino-PEG methyl ester (29 g, 0.025 mol) in 150 mL of DCM and (B) *N*-(9-fluorenylmethoxycarbonyloxy) succinimide or Fmoc-OSu (8.43 g, 0.025 mol) in 50 mL of DCM. Solutions A and B were mixed and allowed to react for 1 h at rt. This was followed by 1.0 mL additions of triethylamine (TEA) until the reaction was complete (a total of 5 mL of TEA was added). The reaction solution was washed with 0.1 M $KHSO_4$ (60 mL) and then with water (60 mL). Both aqueous phases were extracted with DCM (60 mL × 2). The combined organic

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fractions were evaporated under reduced pressure. The resulting crude product **3** was redissolved in HCl (1.5 M) and stirred for 48 h. The solution was then filtered and extracted with DCM (4 × 60 mL). The organic extracts were combined, and the solvent was evaporated (yellow oil), treated with a small aliquot of diethyl ether, and dried giving compound **4** (30 g, 94%, > 98% purity) as a white powder. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.76 (d, J = 7.5 Hz, 1H), 7.61 (d, J = 7.5 Hz, 1H), 7.40 (t, J = 7.5 Hz, 1H), 7.31 (td, J = 7.5, 1.1 Hz, 1H), 4.40 (d, J = 7.0 Hz, 1H), 4.22 (t, J = 7.0 Hz, 0H), 3.70−3.59 (m, 49H), 2.60 (t, J = 6.1 Hz, 1H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 172.9, 156.6, 144.0, 141.3, 127.7, 127.1, 125.1, 120.0, 66.7, 47.3, 41.0, 35.0.

Analysis of Oligomer Purity of Amino-PEG 23 Acid ***1****.* The selected ion monitoring (SIM) method in an Agilent 1269 Infinity II LC/MSD System was used to look for the $n-2$, $n-1$, and $n+1$ impurities. Different injection volumes (0.1, 0.3, 0.6, 1.0, 3.0, 6.0, and 10.0 μL) of a 0.4 μM concentration of amino-PEG acid were used for the analysis. PEG oligomer concentrations (area under the curve of the chromatogram) were plotted against the injection volumes, and a linear range was determined.

Synthesis of Peptides. Peptides were synthesized following a standard Fmoc/*t*Bu-based solid-phase synthesis protocol (SPPS).

Manual Synthesis. Fmoc Rink Amide AM resin (0.71 mmol/g loading, 100−200 mesh) was used as a solid support for the peptides. Initially, the resin was swelled using DMF (3 × 1 min), DCM (3 × 1 min), and DMF (3 × 1 min). Deprotection of the Fmoc group was achieved by treatment of the resin with 20% piperidine/DMF (2 × 5 min) followed by washing with DMF. The first amino acid was coupled to reach a 0.5 mmol/g resin loading (1.5/1.5/1.5 equiv amino acid/DIC/oxyma) for 4 h, then remaining free sites were capped via acetylation treating the resin with acetic anhydride (10 equiv) and DIPEA (10 equiv) in DCM for 15 min. Further residues were incorporated using standard protected Fmoc-amino acids (3 equiv), DIC (3 equiv), and OxymaPure (3 equiv) in DMF, as coupling reagents, for 60 min at rt. To monitor couplings, the Kaiser test was performed. When detecting incomplete reactions, a new coupling was carried out using PyBOP and DIPEA (1.5 and 3 equiv) for 30 min at rt. This was repeated until the final peptide was achieved. Fmoc from the last coupled amino acid was removed as explained above.

Automated Synthesis. Microwave-assisted synthesis was carried out in a Liberty Blue automated peptide synthesizer (CEM Corp., US). Fmoc-removal was carried out with 20% v/v piperidine in DMF at 90°C for 1 min. Couplings were performed mixing 0.125 M of Fmoc amino acids, 0.125 M of OxymaPure, and 0.125 M of DIC at 90 °C for 2 min. Rink Amide ProTide LL resin (0.18 mmol/g loading, 100−200 mesh) was used as solid support. Manual PEGylation was followed using Fmoc-PEG 23 propionic acid and the same coupling agents listed above. For **P13**, Fmoc PEG-11 propionic acid was used. Some peptides were synthesized carrying N-terminal palmitoylation, added as a normal coupling. Cleavage was performed after drying the peptidyl resin by treating with TFA/TIS/H_2O (95/2.5/2.5) for 1 h at rt. The cleavage mixture was then precipitated with cold diethyl ether, centrifuged, and the pellet redissolved in H_2O/ACN (1:1) for lyophilization, after which it was characterized by HPLC and LC−MS.

Purification of Peptides. Crude products were dissolved in either water/acetonitrile (1:1) or DMSO and purified in a semipreparative HPLC Waters PrepLC System (Waters Corp., US) injecting 30−50 mg into either a XBridge Prep C18 OB column (dimensions 19 × 100 mm, particle size 5 μm) or a Kinetex C8 AXI column (dimensions 21.2 × 100 mm, particle size: 5 μm). The monitoring of peptides was done at 220 nm, and elutions were carried in varying gradients of water with 0.1% TFA and acetonitrile with 0.1% TFA, depending on the best resolution for each specific sequence. Data was acquired and analyzed with the Empower 3 software (build 3471, Waters Corp., US). Fractions containing the desired product were manually collected immediately after detection. After the collection and analysis of fractions by analytical HPLC and ESI-MS to confirm purity of 95% or above, the solvent was evaporated by rotavapor and reduced to 20 mL, which was then lyophilized.

Nanoparticle Synthesis. PLGA NPs with encapsulated adjuvants were prepared by using an oil-in-water emulsion and solvent evaporation−extraction method. Briefly, 50 mg of PLGA was dissolved in 3 mL of DCM along with 5 μL of pIC and 2 mg of R848. The solution above was added dropwise to 20 mL of aqueous 2% (w/v) PVA and emulsified for 60 s with 5 s rest each cycle using a sonicator (Sonifier 250, Branson, Danbury, USA). Following overnight evaporation of the solvents at 4 °C, the NPs were collected by centrifugation (14,800 rpm for 30 min) at 4 °C and redissolved in water. After, the nanoparticle solution was added dropwise to 20 mL of 1% homogenized chitosan oligosaccharide lactate solution and stirred at 4 °C for 2 h. The coated NPs were finally collected by lyophilization.

Animals and Immunization. A total of 15 C57BL6 male mice (12 weeks old) were used in the experiment. Mice were obtained and kept at the animal facility of Max-Planck-Institute for Multidisciplinary Sciences under a 12 h dark: light cycle with ad libitum access to food and water. All animal experimental procedures were performed in compliance with the European (2010/63/EU) and German regulations on Animal Welfare and were approved by the administration of Lower Saxony (LAVES) (Nr. 33.19-42502-04-20/3527). Mice were immunized subcutaneously in the right flank once per week, for 3 weeks with nanoparticle containing the adjuvants polyinosinic:polycytidylic acid (polyIC) and R848. The NPs were diluted in water and injected in a volume of 100 μL, in a concentration of 2.5 μg of polyIC, and 3.75 μg of R848. The mice were sacrificed 2 days after the last vaccination, and the spleen was excised.

Stimulation of Splenocytes. Single-cell suspensions from the spleen were prepared in sterile conditions by mincing the cells through 40 and 100 μL of cell strainers (BD Falcon). Erythrocytes in spleen samples were lysed with 1 mL of ACK buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0,1 mM EDTA, pH = 7.2−7.4) per spleen for 5 min. The reaction was stopped with PBS and spun down. Cells were resuspended in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin. Cells were seeded in 96-well plates with a density of 500,000 cells per well in duplicates or triplicates. Splenocytes were stimulated with concanavalin A (2 μL/mL, 00-4978-03, ThermoFisher) or different peptides in a concentration of 10 μg/mL. The supernatant was collected after 48 h of stimulation and kept frozen at −20 °C until cytokine analysis.

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After 48 h of stimulation, the cells were analyzed for their viability.

ELISA. Cells were centrifuged, and supernatants were collected and kept at −20 °C for further cytokine quantification. IFN-γ was quantified by ELISA (Thermoscientific, #88-7314-88). Briefly, the 96-well plates were incubated overnight at 4 °C with the capture antibody. After being washed and blocked, the plate was incubated at room temperature with samples for 2 h and then with detection antibody for 1 h. After that, the plate was incubated with the avidin horseradish peroxidase (HRP) conjugate for 30 min. The color reaction was developed by adding TMB solution, and the enzymatic reaction was stopped by adding 2 N H_2SO_4. Optical density (OD) was determined to be 450 nm.

Cell Viability Assay. For cell proliferation and cytotoxicity assay, CellTiter 96 AQueous One Solution Reagent (Promega) was used. The reagent contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; MTS] and phenazine ethosulfate; PES. After 48 h of stimulation with the peptides, 20 μL of the reagent was added to each well, and the plate was incubated for 4 h. The absorbance at a wavelength of 490 nm was measured by a microplate reader.

Statistical Analysis. Results are expressed as mean ± SEM (standard error of the mean). Data were analyzed, and statistical analyses were performed using the GraphPad Prism v9.2.0 software. Each experiment was performed three times with triplicates per assay. Data are presented as mean ± SEM. One-way analysis of variance (ANOVA) with Tukey's test for multiple comparisons was performed. A p-value of <0.05 was considered statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c02604.

Synthesis of Fmoc-PEG 23 propionic acid along with ^{1}H and ^{13}C NMR spectra; characterization of peptides; chromatogram (crude and purified) and MS spectrum of synthesized peptides (PDF)

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Notes

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■ ABBREVIATIONS

PEG: polyethylene glycol; SPPS: solid-phase peptide synthesis; PDAC: pancreatic ductal adenocarcinoma; MSLN: mesothelin; ELISA: enzyme-linked immunosorbent assay concentration; HPLC: high-performance liquid chromatography; MS: mass spectroscopy; NMR: nuclear magnetic resonance

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