Thiopalmitoylation of altered peptide ligands enhances their protective effects in an animal model of multiple sclerosis.

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Abstract

Previously we have shown that conjugation of a palmitic chain via a thioester bond to a cysteine residue in weakly- or non-encephalitogenic or neuritogenic peptides markedly enhances their ability to induce autoimmune disease in an MHC class II restricted manner. From those studies, however, it was not clear whether thiopalmitoylation of the peptides was merely enhancing their disease-inducing potential, or whether the lipid was itself playing a pathogenic role. To investigate this further, we have now tested the effects of thiopalmitoylation on MHC class II-restricted altered peptide ligands (APLs), which are normally protective in experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis. We hypothesized that if thiopalmitoylation of a peptide merely enhances its innate potential, then thiopalmitoylated APLs (S-palmAPLs) should show enhanced protective effects. Alternatively, if thiopalmitoylation itself can make a peptide pathogenic, then S-palmAPLs should have decreased therapeutic potential. We synthesized APLs and corresponding S-palmAPLs, and showed that the S-palmAPLs were much more effective than the non-conjugated APL at inhibiting the development of EAE. This was due to several features of the S-palmAPL: S-palmAPL-primed cells show an enhanced ability to proliferate and produce the anti-inflammatory cytokine, IL-10, *in vitro*. Furthermore, the bioavailability of S-palmAPL was greatly enhanced, compared to the non-palmitoylated APL, and S-palm APL was taken up more rapidly into DCs and channelled into the MHC class II processing pathway. These results show that thiopalmitoylation of MHC class II restricted peptides is a simple way to enhance their effects *in vivo,* and could have wide therapeutic application.

Introduction

The role that lipid post-translational modifications to proteins play in the immune response to those proteins is still poorly understood. We have been interested in the role that one such modification, thiopalmitoylation (attachment of palmitic acid via a thioester bond to cysteine residues of a protein), might play in immune responses directed against myelin proteolipid protein (PLP), a major component of CNS myelin and a putative autoantigen in the demyelinating disease, multiple sclerosis (MS). PLP is normally thiopalmitoylated at up to 6 sites, at a ratio of approximately 3 moles of lipid per mole of protein; however, it is known that the degree of thiopalmitoylation of PLP increases markedly during the process of demyelination (1), and thus, during myelin breakdown in MS, there is the potential for thiopalmitoylated PLP peptides to be released. Previously, we have shown that thiopalmitoylation of specific PLP peptides markedly enhances their encephalitogenicity in an experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis (MS) (2). Similarly, thiopalmitoylation of peptides from P_0, a major protein of peripheral nervous system myelin, enhances their neuritogenic potential in experimental autoimmune neuritis (EAN) (3). The enhanced pathogenicity of the thiopalmitoylated peptides appears to be due to their increased uptake (compared to non-palmitoylated peptide) into the MHC class II presentation pathway (4, 5). One question arising from the above studies is whether the thiopalmitoylation is merely enhancing the innate potential of these peptides to be pathogenic, or whether thiopalmitoylation of peptides that have little or no disease-inducing potential can actually make them pathogenic. In order to address this question, we decided to test the effects of thiopalmitoylation on MHC class II-restricted altered peptide ligands (APLs) that are normally protective in EAE. We hypothesized

that if thiopalmitoylation of a peptide merely enhances its innate potential, then thiopalmitoylated APLs (S-palmAPLs) should show enhanced therapeutic efficacy. Conversely, if thiopalmitoylation itself can make a peptide pathogenic, then S-palmAPLs should have decreased therapeutic potential and/or should become pathogenic.

APLs are analogs of immunogenic peptides that have been modified at one or more key TCR contact positions in order to inhibit or modulate the response of T cells that recognise the cognate ligand from which the APLs are derived (6, 7). Depending on their particular sequence, APLs can i) induce responses in T cells similar to those induced by the cognate ligand, ii) induce some, but not all, of the signals and effector functions downstream of the TCR that are generated by the cognate ligand, leading to altered patterns of cytokine production or to anergy, or iii) antagonise T cell activation by generating a dominant negative signal (6, 8-13). APLs with antagonistic and immunomodulatory properties have the potential to therapeutically suppress pathogenic T cell-mediated autoimmune responses.

In experimental animals, APLs have been found to effectively and reliably dampen autoimmune responses, particularly when used at high concentrations (8, 9, 14). Several well-defined APLs have been derived from the immunodominant epitopes of PLP for SJL mice, PLP$_{139-151}$ and PLP$_{178-191}$ (15, 16). For PLP$_{139-151}$, two APLs have been studied in detail, namely Q144, which has a substitution of glutamine (Q) for tryptophan at position 144 (9), and the double substituted APL, L144R147 (leucine at position 144 and arginine at position 147 (8, 9). The APL related to PLP$_{178-191}$ is known as A188 and has an alanine (A) at residue 188 instead of phenylalanine

(14). Both Q144 and A188 appear to exert their *in vivo* effects predominantly through increasing levels of the cytokines IL-4, IL-10 and IFN-γ (9, 14).

In the current study, we compared the effects of non-palmitoylated APLs (A188 and Q144) and the corresponding S-palmAPLs to determine whether or not S-palmAPL are themselves encephalitogenic and lose the therapeutic efficacy of the non-palmitoylated APLs, or whether the protective effects of the APL are enhanced by thiopalmitoylation. We show that S-palmAPLs show greatly enhanced protective effects, and that this appears to occur because of the effects of thiopalmitoylation on stability of the peptide in serum, increased uptake of the APL into the MHC class II presentation pathway, and induction of greatly enhanced levels of IL-10 following S-palmAPL administration. The enhanced levels of IL-10 also appear to induce bystander effects, enabling S-palmAPL to inhibit the encephalitogenicity of unrelated peptides *in vivo*.

Materials and Methods

Peptide Synthesis

Peptides (Table 1) were manually synthesized by solid phase-synthesis using the Fmoc/tBu strategy. Thiopalmitoylation of residue Cys at position 183 was performed on the resin-bound peptide after selective deprotection of the Cys(Mmt) side chain (17). The biotin-labelled thiopalmitoylated peptides were synthesized as we have previously described (4). After cleavage from the resin, the crude peptides were lyophilized and purified by reverse phase HPLC (RP-HPLC). The purity of the peptides was assessed by analytical HPLC and their identities were confirmed by MALDI-TOF mass spectrometry.

For injections into mice and for *in vitro* tissue culture, stock solutions of 5 mg/mL peptides dissolved in 0.2M acetic acid were further diluted in phosphate-buffered saline (PBS) or tissue culture medium immediately prior to use. For the uptake experiments, stock solutions of pure biotinylated peptides were made up at a concentration of 5 mg/mL in H_2O (A188-Biot), or in 30% DMSO (S-palmA188-Biot).

EAE induction and clinical assessment

SJL/J mice were purchased from the Animal Resources Centre (Murdoch, Western Australia), and were immunized at 6-8 weeks of age. Mice were maintained and used in accordance with the University of Queensland ethical guidelines for use of experimental animals, and the experimental protocol was approved by the Animal Ethics Committee of the University of Queensland.

For induction of EAE, 32-65 μmoles of peptide and 400 μg of *M. tuberculosis* H37Ra (Difco, Detroit, MI, USA) in an emulsion consisting of equal volumes of PBS and CFA was injected subcutaneously on the back of the mouse. Each mouse also received 300 ng of *Bordetella pertussis* toxin (Sapphire Biosciences, Redfern, NSW, Australia) i.v on day 0 and day 3. Clinical assessment (weighing & observation) was conducted daily from day 7 post-injection. Mice were scored according to the following criteria: 0, no disease; 1, decreased tail tone or slightly clumsy gait; 2, tail atony and/or moderately clumsy gait and/or poor righting ability; 3, hind limb weakness; 4, hind limb paralysis; 5, moribund state. For co-immunisation experiments, APLs or S-palmAPLs, at different molar ratios, were included in the emulsion with the adjuvant.

T cell proliferation assays

Mice were immunised s.c. with 32 μmoles of PLP$_{178-191}$, A188, or S-palmA188 peptide in an emulsion consisting of equal volumes of PBS and CFA (Difco). Ten days after immunisation, mice were euthanized, and axillary and inguinal lymph nodes were removed and teased apart. Lymph node cells (LNC) pooled from at least 2 mice were washed twice with sterile PBS and stained with 2 μM Carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, Life Technologies, Melbourne, Australia) for 30 min at 37 °C. Cells were cultured at $4x10^6$/mL in phenol red-free RPMI 1640 (Life Technologies) containing 10% Serum Supreme (Lonza Australia, Melbourne, Australia), 2mM HEPES (Lonza), 50μM 2-ME (Sigma-Aldrich, Sydney, Australia), and 2mM l-glutamine (Lonza), in the presence or absence of antigen (25 μg/mL) for 5 days. Wells with no antigen and ConA (2 μg/mL) were used as negative and positive controls, respectively.

After 5 days, cells were harvested and washed with PBS containing 1% FCS and 0.01% sodium azide (Sigma) (wash buffer), before staining with Per-CP-labelled anti-CD4 or anti-CD8 antibodies (BD Biosciences, Sydney, Australia) for 1h at 4°C in the dark. Per-CP-labelled isotype-matched primary antibodies (BD Biosciences) were used as controls. Stained cells were analysed on a FACSCalibur flow cytometer using CellQuest Software (BD Biosciences). All staining profiles were based on lymphocyte-gated cells, as determined by forward and side scatter properties. The results are expressed as a percentage of $CD4^+$ or $CD8^+$ cells in the sample that are dividing (reactive) in response to the antigen, and a cell division index (CDI) was calculated by dividing the percentage of dividing cells in the antigen-stimulated group by the percentage of dividing cells in the control group (no antigen). Assays were done in triplicate.

Cytokine ELISA

Culture supernatants were collected after 72 h from LNC cultured with antigen as for proliferation assays, and stored frozen at -80°C until use. Ready-Set-Go sandwich ELISA kits (eBiosciences, San Diego, CA) specific for IL-10, IL-17, and IFN-γ were used to determine the concentration of cytokines in the supernatants, as per the manufacturers' instructions. Each supernatant was tested in triplicate, and the concentrations of cytokines were determined from comparison against standard curves prepared from serial dilutions of recombinant cytokines tested on the same plate as the test samples. Results from duplicate assays were calculated and graphed as a fold change in concentration of stimulated cells compared to unstimulated/background (no antigen) levels.

Cytokine Bead Array (CBA) assay for measuring levels of IL-10 and IFN-γ

Levels of the cytokines IL-10 and IFN-γ in culture supernatants from control or antigen-stimulated LNC of mice coimmunized with PLP139-151 and A188 or S-palmA188 were quantitated using CBA Mouse IL-10 (bead C4) and IFN-γ (bead A4) sets (BD Biosciences, Franklin Lakes, NJ, USA), as per the manufacturer's instructions. Briefly, undiluted culture supernatant was incubated with cytokine capture antibody-bead complexes and PE-conjugated antibody (specific for beads) for 2hrs. Data was acquired using a BD FACScalibur flow cytometer and analysed using FCAP software (BD Biosciences). The concentrations of each cytokine (pg/ml) were determined from a standard curve generated using 10 serial dilutions of cytokine standards.

Stability of APL and S-palmAPL in serum

Peptide (500 μg) was dissolved in 900 μL of 0.05 M phosphate buffer (pH 7.2) containing 1% acetonitrile. Fetal calf serum (10% serum, v/v) was added (100 μL). The reaction mixture was incubated at 37 °C. At different incubation times, a 100 μL aliquot was collected, and protease activities were blocked by adding 2 vol of acetonitrile (200 μL). After centrifugation at 3000 rpm for 5 min, the supernatant was analyzed by RP-HPLC. In these conditions, no peptide precipitated. A control sample was run in the absence of peptide.

Uptake and presentation of APL and S-palmAPL by DC

Dendritic cells (DC) were isolated from spleens of SJL/J mice (Janvier, Le Genest-Saint-Isle, France) using positive selection with $CD11c^+$ MicroBeads (N418) (Miltenyi Biotec, Paris, France) as per the manufacturer's instructions. DC were resuspended in RPMI medium containing 5% FCS and 5ng/mL GM-CSF and allowed to adhere to glass coverslips for 2hrs at

37 °C. Non-adhered cells were washed off and DC were then incubated with 100 μM of biotinylated peptide for between 5min to 1 h at 37°C. After incubation, cells were fixed with 4% formaldehyde in PBS for 15 min at room temperature and permeabilized with 0.2% triton X100 in PBS for 5 min at room temperature.

For assessment of the amount of peptide taken up by DC, DC were next incubated with streptavidin-Alexa 488 (1/400 dilution in PBS) (Invitrogen, Cergy Pontoise, France) to detect the biotinylated peptide. In experiments to assess MHC class II colocalization, cells were incubated after fixation and permeabilization with Anti-Mouse I-A^p (anti-MHC class II; cross reacts with I-A^s) (FITC labelled) (1/100 dilution in PBS) (BD Biosciences, Le Pont de Claix, France) overnight at 4°C and then with Streptavidin-Cy3 (1/400 dilution in PBS) (Invitrogen) at room temperature for 45 min. After staining, cells were washed in PBS, and coverslips were mounted using Aquapolymount medium. Immunofluorescence staining was monitored with a laser scanning microscope (Leica SP5 II) equipped with a Leica HCX PL APO 63x oil DIC immersion lens (numerical aperture 1.4-0.6).

Uptake of APL and S-palmAPL was also compared using flow cytometry. DC in suspension were incubated with 10μM of the different biotinylated peptides for 10 min at 37 °C. At the end of the incubation, cells were washed and incubated with anti-CD11c (APC labelled) and anti-I-A^p (FITC labelled) (1/300 dilution) (BD Biosciences) to detect dendritic cells. After washing, the cells were fixed and permeabilized with BD Cytofix/Cytoperm and incubated with PE-streptavidin to detect the biotinylated peptides. The cells were analyzed by flow cytometry using

a FACSCalibur system (Becton Dickinson). Samples were gated on the $CD11c^{+}/I\text{-}A^{p+}$ population, and the mean fluorescence intensity of staining with PE-streptavidin was determined.

Statistics

Most statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, California, USA). Normality tests were initially performed to determine whether or not the data was normally distributed. Differences between more than two groups were tested using ANOVA (for parametric data) or the Kruskal-Wallis test (for nonparametric data). If analysis of the group as a whole showed a significant difference ($p<0.05$), then appropriate post-tests (Dunnett's test with ANOVA or Dunn's Multiple Comparisons test with Kruskal-Wallis) were used to compare pairs of groups. For analysis of the course of EAE, multiple time point 2-way ANOVA was used. For comparison of disease incidence in mice, χ^2 analysis with Yates' correction was used. The log-rank (Mantel Cox) test was used to compare survival curves (showing percentage of mice free of EAE).

Results

Effects of thiopalmitoylation of APLs on their protective effects in vivo

The APL A188 is derived from the encephalitogenic peptide PLP178-191, by substituting an alanine (A) for phenylalanine at position 188. A188 is not encephalitogenic *in vivo* and is able to protect against the induction of PLP178-191-induced EAE in SJL/J mice when it is used at a higher molar ratio than the inducing peptide (14). A188 and S-palmA188 were synthesized by solid phase-synthesis (Table 1); the palmitic chain was linked via a thioester bond to Cys183 in the S-palmA188 peptide. We then compared the effects of A188 and S-palmA188. Initial studies showed that neither A188 nor S-palmA188 were encephalitogenic when the peptides (emulsified in CFA) were injected into SJL/J mice (Table 2). Next, mice were co-immunized with mixtures of encephalitogenic PLP178-191, together with either A188 or S-palmA188 at various molar ratios, and followed for development of EAE. The protective effect of A188 was significantly enhanced by thiopalmitoylation, as evidence by decreased incidence of disease, increased mean day of onset, and decreased mean severity of EAE (Table 2 and Figure 1). When S-palmA188 was injected at a ratio of 1:1 with PLP178-191, animals were completely protected from disease; in contrast, EAE in mice treated with non-palmitoylated A188 at a 1:1 ratio with PLP178-191 did not differ significantly from mice injected with PLP178-191 alone (Table 2). In addition, a substantially smaller quantity of S-palmAPL than A188 was required to induce the same clinical effect, with 1:0.1 PLP178-191:S-palmA188 treatment giving the same outcome as 1:5 PLP178-191:A188 (Figure 1).

In order to ensure that the protective effects observed were not restricted to a single APL, we

also tested the APL Q144, derived from the encephalitogenic peptide PLP139-151 (Table 1). Previous work has shown that Q144 induces a highly Th2 polarized response and is effective at inhibiting EAE induced by PLP139-151 at a 1:6 ratio of PLP139-151:Q144 (9). As shown in Table 2, the protective effect of Q144 was also enhanced when the peptide was palmitoylated on Cys150.

Effects of A188 and S-palmA188 on T-cell proliferative responses

We have previously shown that the APL A188 protects against EAE primarily via induction of A188-specific $CD4^+$ Th0/Th2 cells (14). To determine if a stronger A188-specific $CD4^+$ T cell response was induced in mice immunized with the thiopalmitoylated peptide, we next compared the proliferation of $CD4^+$ and $CD8^+$ T cells from mice immunized with A188, S-palmA188, or PLP178-191, in response to PLP178-191 or A188. We have used A188 to stimulate T cells generated in mice immunized with either A188 or S-palmA188, as we have shown in a previous study that the lipid tail is cut off the peptide within the endosomes or lysosomes of the antigen-presenting cells, due to the presence of thioesterases in these organelles (5); thus, the T cells would recognize only the A188 portion of the molecule, irrespective of whether the antigen-presenting cells take up non-palmitoylated peptide or thiopalmitoylated peptide.

LNC from mice immunized 10 days previously with A188, S-palmA188, or PLP178-191 were stained with CFSE and stimulated with antigen *in vitro* for 5 days. LNC were subsequently stained with anti-CD4 or anti-CD8 antibodies and analysed to assess cell proliferation. Very few proliferating $CD8^+$ cells were detected (data not shown). $CD4^+$ T cells proliferated in an antigen-specific manner (Table 3). Most notably, the percentage of proliferating A188-specific T cells

was twice as high in LNC from mice immunized with S-palmA188 (10.7% ± 2.6%) than in LNC from mice immunized with A188 (5.2% ± 1.5%), showing that thiopalmitoylation efficiently enhances the proliferation of $CD4^+$ T cells. PLP178-191-specific T cells proliferated minimally following stimulation with A188, but proliferated strongly in response to PLP178-191. There were no significant differences in the ability of the LNC from mice immunized with A188 or S-palmA188 to proliferate in response to PLP178-191, or to the unrelated PLP139-151 peptide.

Cytokine production induced by immunization with A188 and S-palmA188

Previously we have reported that A188-specific T cells have elevated levels of mRNA for the cytokines IL-4, IL-5, IL-10 and IFN-γ, compared to controls, and have speculated that the APL prevents EAE by inducing the production of Th0 and Th2 cytokines by A188 specific cells (14). Since the time when those experiments were done, Th17 cells have been shown to play an important role in the development of EAE (18). To evaluate the effect of thiopalmitoylation of A188 on the production of cytokines we measured levels of 3 key cytokines (IFN-γ, IL-10 and IL-17A). Culture supernatants were collected from 72 h *in vitro* cultures (in the presence of no antigen, PLP178-191 or A188) of LNC from mice that had been immunized with PLP178-191, A188, or S-palmA188. Significant differences were seen amongst the different groups with respect to production of IFN-γ, IL-10, and IL-17A (Figure 2).

Following *in vitro* stimulation with PLP178-191, LNC from mice immunized with PLP178-191 showed a greater than 500-fold increase in the amount of IL-17A produced, and smaller fold changes in levels of IFN-γ and IL-10. After *in vitro* stimulation with A188, these same LNC increased production of IL-10, but showed minimal increases in levels of IFN-γ and IL-17. In

contrast, LNC from mice immunized with A188 or S-palmA188 cells showed no increases in the amounts of IL-17 when stimulated *in vitro* with either A188 or PLP178-191. A188-specific and S-palmA188-specific LNC both increased production of IFN-γ in response to A188 stimulation, but not when stimulated with PLP178-191. The most significant finding from this experiment was the greatly enhanced ability of S-palmA188-specific LNC to produce the anti-inflammatory cytokine IL-10 following stimulation with A188 (P<0.001 compared to other groups).

S-palmA188, but not A188, can induce bystander suppression of PLP139-151-induced EAE.

Because S-palmA188 was able to induce production of significantly higher levels of the anti-inflammatory cytokine IL-10 than could A188, we next tested whether some of the effects of the S-palmA188 might be due to bystander suppression of pro-inflammatory responses in a non antigen-specific manner. To test this, mice were immunized with 32 μmoles of PLP139-151 alone, 32 μmoles PLP139-151 + equimolar amount of A188, or 32 μmoles PLP139-151 + equimolar amount of S-palmA188. Mice were then followed for 35 days. All mice immunized with PLP139-151 developed EAE, although since the amount of PLP139-151 used to induce disease was less than half of the amount used for disease induction in the mice in Table II, the average day of onset of EAE was sightly later than that shown in Table II (Figure 3A) and the mean severity was lower (2.8 ± 0.4). Three of four mice coimmunized with PLP139-151 and A188 also developed EAE with the same kinetics of disease (Figure 3A). In contrast, only two of eight mice coimmunized with PLP139-151 and S-palmA188 developed EAE (Figure 3A). In the 3 A188-coimmunized and 2 S-palmA188-coimmunized mice that did develop EAE, disease severity was not significantly less than that seen in the mice immunized with PLP139-151 alone. These results

strongly suggest that a bystander response induced by S-palmA188 was sufficient to overcome the encephalitogenicity of PLP139-151.

To investigate whether this non antigen-specific protection might be due to the production of IL-10 and/or IFNγ, LNC were removed from the above mice on day 35 after immunization. At the time when LNC were collected, all mice were in the recovery or remission phase of disease. Cultures were set up from each individual mouse's LNC to assess the production of IL-10 and IFN-γ upon stimulation of the LNC with either no antigen, PLP139-151 or A188. After 3 days in *vitro* stimulation, culture supernatants were harvested and the levels of IL-10 and IFN-γ in the supernatants were assessed using CBA assays. All mice were found to make a low level of IL-10 upon stimulation with PLP139-151 (Figure 3B). However, only LNC from mice coimmunized with either A188 or S-palmA188 made significantly increased amounts of IL-10 upon stimulation with A188 (Figure 3B). A188-coimmunized mice had a 12-fold increase in IL-10 levels, compared to the no antigen group. In S-palmA188-coimmunized mice, there was a 19.4-fold increase in IL-10 levels. Significant fold changes in levels of IFN-γ were also seen in the mice co-immunized with either A188 or S-palmA188 (3.6 fold increase in each, compared to no antigen group). These results support the proposition that the production of significant amounts of IL-10 by cells responding to S-palmA188 can act non-specifically to decrease the encephalitogenic response normally induced by PLP139-151. Since A188 co-immunized mice also produced reasonable amounts of IL-10, it may be that there is a threshold amount of IL-10 required to induce protection from EAE, which was not quite reached in the A188-treated animals.

Stability of A188 and S-palmA188 in the presence of serum

Another potential reason for the enhanced effectiveness of S-palmA188 *in vivo* could relate to its bioavailability. We therefore next investigated the stability of A188 and S-palmA188 in the presence of serum. For this purpose, both peptides were incubated in phosphate buffer in the presence of 10% FCS, and the progress of the digestion was followed by HPLC. As shown in Figure 4, the S-palm peptide exhibited greater stability than the non-palmitoylated peptide, since most of the A188 was digested after 3 h, while 85% of S-palmA188 remained undigested at that time point. The half-life of S-palmA188 was significantly longer (>850 min) than that of A188 (50 min), which would enhance the bioavailability of S-palmA188.

Uptake of A188 and S-palmA188 by DCs

DCs are the professional APC of the immune system. Previously, we have found that thiopalmitoylated encephalitogenic peptides are not only taken up more efficiently by another type of APC (macrophages) than are the same non-palmitoylated peptides, but are also channelled into the MHC class II presentation pathway, and we have proposed that this underlies their improved immunogenicity and the enhancement of $CD4^+$ T cell responses (4, 5). In this study, we sought to confirm whether S-palmAPL was taken up more efficiently than non-palmitoylated APL by DCs.

DCs were purified from spleens of SJL/J mice and incubated for 10 min in the presence of biotinylated S-palmA188 or non-palmitoylated A188 (Table 1). PE-conjugated streptavidin was used to detect the peptide taken up by these cells by flow cytometric analysis. Thiopalmitoylation enhanced the uptake of APL by DCs, as indicated by higher levels of PE

staining (Figure 5A). This was further confirmed using confocal microscopy on purified splenic DCs incubated with the APL and S-palmAPL for different times (Figure 5B-F). S-palmA188 could be easily detected at the cellular membrane after 5 min (Figure 5B), and after just 15 min the translocated peptide showed a punctate fluorescence staining pattern, indicative of localization in discrete vesicular compartments and suggestive of an endocytic pathway of internalization (Figure 5 C-E). In contrast, even after 60 min, hardly any non-palmA188 peptide had been taken up into DCs (Figure 5F). These results confirm that the palmitic chain markedly enhances the efficiency of peptide translocation into DCs.

We have previously demonstrated with encephalitogenic thiopalmitoylated peptides that the thioester bond between the lipid and peptide can be broken down in endosomes and lysosomes (part of the MHC class II presentation pathway and which contain thioesterases), thereby effectively "stranding" the peptide in these organelles, and making it much more likely that they will be presented by MHC class II molecules (5). In order to assess if this same mechanism was likely to be occurring with the S-palmA188 peptide, we investigated whether there was co-localization of biotinylated peptide and MHC class II in DCs that has been incubated with the biotinylated peptide for 60 min. Most of the biotinylated peptide (detected using Streptavidin-Cy3) co-localized with MHC class II (Figure 5G). These results confirm that the thioester bond between lipid and peptide helps to channel the peptide into the MHC class II presentation pathway, due to the presence of thioesterases in these organelles, thereby inducing a $CD4^+$ T cell response, as we have previously found with encepalitogenic peptides in macrophages (5).

Discussion

In the current study, we have shown that thiopalmitoylation of APLs can enhance their protective properties. Using a co-immunisation protocol, we showed that thiopalmitoylation greatly enhanced the prophylactic abilities of the A188 APL (Figure 1 and Table 2), with a 50-fold lesser quantity of S-palmA188 needed to induce the same protective effects as non-palmitoylated A188. The complementary data obtained from S-palmQ144 show that the enhancement of the protective effects afforded by thiopalmitoylation is not exclusive to one APL, but can be generalized to other APLs. These results support the idea that thiopalmitoylation of a peptide enhances its innate properties; thus, thiopalmitoylation of a peptide that has the potential to be pathogenic can enhance pathogenicity (as we have previously shown in EAE (2, 5), and EAN (3)), whereas thiopalmitoylation of a peptide that has the potential to protect against disease can enhance the efficacy of the therapeutic effect. This could have potential applicability in vaccine development, providing a simple means to increase MHC class II restricted T cell responses.

There appear to be several mechanisms that are operative in the enhancement of immunogenicity of the A188 APL by thiopalmitoylation. Firstly, thiopalmitoylation greatly enhanced the stability and longevity of A188 peptide in serum, with S-palmA188 having a half-life more than 15 times that of the non-palmitoylated APL in the presence of serum (~875 min vs 42 min, respectively). This could be due to formation of micelle-like aggregations or particles, as has previously been reported for N-palmitoylated peptides (19, 20). The characteristic of enhanced stability in serum is yet to be confirmed for other S-palm peptides; however, the increase in bioavailability is almost certainly one element important for the enhanced immunological activity and *in vivo* effects of S-palmA188.

Secondly, uptake of S-palmA188 compared to A188 into the class II MHC presentation pathway of DC was greatly enhanced. Transient thioacylation, particularly by attachment of myristic acid (C14) or palmitic acid (C16), is a common mechanism that cells use to move molecules through membranes; this can occur via non-phagocytic mechanisms, endocytosis or patocytsis [reviewed in (21)]. Our previous work suggests that thiopalmitoylation of PLP peptides allows uptake via non-phagocytic mechanisms (5). Furthermore, using S-palm peptides in which the palmitic acid is replaced by a fluorescent lipid analogue, we have previously shown that the lipid moiety is cleaved from the peptide within endosomes, thereby stranding the peptide moiety in the endosome (5); this would account for the preference for the class II MHC pathway, as the endosomes are part of this pathway.

Lastly, S-palmA188-primed cells, compared to those primed by A188, showed an enhanced ability to proliferate (Table 3) and to produce the anti-inflammatory cytokine IL-10 (Figure 2) in the presence of APL stimulation; this can explain the increased efficacy of S-palmA188 *in vivo* compared to non-conjugated A188. Interestingly, both A188- and S-palmA188-primed cells produced copious amounts of IFN-γ, a cytokine often regarded as pro-inflammatory; however, it is known that IFN-γ can inhibit Th17 responses (22, 23) and, furthermore, several studies have reported more severe and chronic disease in anti-IFN-γ antibody treated (24) and IFN-γ gene knock-out (25) EAE models. Taken together, these findings support the idea that IFN-γ can cause down-regulation of pathogenic Th17 mediated disease (26). It is also of interest to note that development of mucosal tolerance to another myelin antigen, MBP, has recently been

reported to be mediated by a Th0 cell population producing both IFN-γ and IL-10 (27), similar to the phenotype of the S-palmA188-specific cells reported here.

Because of the induction of copious amounts of IL-10 in mice immunized with S-palmA188, we also tested the potential of S-palmA188 to protect against induction of EAE with an unrelated antigen. As shown in Figure 3, coimmunization with S-palmA188, but not A188, effectively protected against disease induced by PLP139-151. This protection correlated with enhanced production of IL-10 in the mice co-immunized with PLP139-151 and S-palmA188. There was also an elevated level of IL-10 produced in mice co-immunized with PLP139-151 and A188, although not to extent of that induced by S-palmA188. These results suggest that S-palmAPLs could potentially have both specific and non-specific bystander therapeutic effects: in a therapeutic setting, this could be a distinct advantage.

A small number of human APL trials have been undertaken in patients with autoimmune disease (28-32); they have not been very successful thus far, although this has been due largely to trial design issues such as poor selection of APLs (using APLs that were capable of acting as full agonists for some patient T cell clones (33, 34)) and of patients (not screening patients to ensure that they carried the specific HLA molecules for which the APLs were designed). However, part of the lack of success of the human APL trials also stems from the poor *in vivo* stability and low immunogenicity of peptides, and the severe injection-site reactions that developed when very large amounts of peptide were administered to try to overcome these issues. The findings in the current study suggest that S-palmAPL may have significant advantages over APLs in future

human trials, given their markedly increased stability and immunogenicity, provided APL- and patient-selection issues are adequately addressed.

Thiopalmitoylation of other types of peptide vaccines may also help to enhance $CD4^+$ T cell mediated prophylactic or therapeutic effects. For example, current studies are trying to find a way to enhance $CD4^+$ T cell responses to cancer vaccines, so as to promote more robust priming and long-term protective $CD8^+$ T cell responses (35). Thiopalmitoylation of peptides may be one way to achieve this. The protective effects of the S-palmAPLs do not appear to be dependent on the position in the peptide chain of the cysteine residue to which the palmitic chain is linked via a thioester bond, as this was the second residue for PLP139-151 and the sixth residue for PLP178-191. If a peptide does not contain a cysteine in its sequence, it may be possible to replace an existing serine with a cysteine without affecting the biological activity (3), or to add a cysteine at the N- or C- terminus of the peptide.

Taken together, our results suggest that the strategy of thiopalmitoylation of a peptide has great potential to enhance $CD4^+$ T cell responses to that peptide. The lipid group does not change the type of response that is induced; it merely enhances the type of response that the peptide has the capacity to induce. We provide evidence that S-palmAPL can be highly efficient at a much lower dose than the non-palm APL. This strategy could effectively and safely enhance APL therapy for many autoimmune and inflammatory diseases, including MS, diabetes type 1, rheumatoid arthritis and myasthenia gravis. Thiopalmitoylated peptides also have the potential to enhance immunogenicity of peptide vaccines that aim to induce a $CD4^+$ T cell response.

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Figure Legends

Figure 1. A) Clinical course of EAE in mice immunized with PLP178-191, PLP178-191 + A188 (at 1:5 ratio), or PLP178-191 + S-palmA188 (at 1:1 and 1:0.1 ratios). At 1:1 ratio, the S-palmA188 completely prevented onset of EAE ($P<0.0001$ compared to PLP178-191 at days11-17 and $P<0.01$ at day 18). S-palmA188 at 1:0.1 ratio was as effective as A188 at 1:5 ratios; both of these were significantly less than the PLP178-191 group at days12-18. B) The percentage of mice free of EAE. All mice in the PLP178-191 group had developed EAE by day 14 after induction of EAE. All mice in the S-palmA188 (1:1) group remained free of disease, which was highly significantly different from the PLP178-191 group ($P<0.0001$). Around 60% of the mice in both the A188 (1:5) and S-palmA188 (1:0.1) groups remained free of EAE (*P* values given next to these lines are for the comparison with the PLP178-191 group).

Figure 2. Production of cytokines (IFN-γ, IL-10, IL-17) by LNC from mice immunized with PLP178-191, A188 or S-palmA188 following stimulation with PLP178-191 (black bars) or A188 (stippled bars), as indicated by fold change compared to unstimulated cells. Background levels of cytokines were: IFN-γ - 37.5 ± 10.5 pg/ml; IL-10 - 54.3 ± 17.6 pg/ml; IL-17 - 5.1 ± 0.9 pg/ml.

Figure 3. S-palmA188 can induce non antigen specific protection in the coimmunization model, probably through induction of a Th0-like response. A) Mice were immunized with PLP139-151 alone or coimmunized with PLP139-151 together with A188 or S-palmA188. The percentage of mice free of EAE is shown. All mice immunized with PLP139-151 alone (n=8) developed EAE, as did 3 of 4 mice coimmunized with PLP139-151 and A188. In contrast, only 2 of 8 mice

coimmunized with PLP139-151 and S-palmA188 developed EAE, which was significantly different to the control group (P=0.0002 by the Log-rank (Mantel-Cox) test). B) At day 36 after the commencement of the coimmunization experiment, lymph node cells were removed from each mouse and activated *in vitro* with no antigen, PLP139-151 or A188. After 72 hours, supernatants from each culture were harvested and subsequently tested for production of IL-10 and IFN-γ in CBA assays. The levels of cytokines (mean ± SE for each group of mice) are expressed as pg/ml.

Figure 4: Kinetics of digestion of A188 and S-palmA188 in the presence of serum. The dotted lines show the half-lives ($t_{1/2}$) of each peptide.

Figure 5: The uptake of A188 and S-palmA188 peptide by splenic DCs. (A) Peptide uptake after 10 min indicated by fluorecent intensity measured via flow cytometry. (B-F) Confocal microscopy analysis of uptake of biotinylated peptides (stained green): B-E) Uptake of S-palmA188 after the indicated times or F) uptake of A188 after 60 min. G) Colocalization of MHC class II and peptide indicated by merging (yellow) of MHC class II staining (green) and peptide staining (red). Scale bar 5 μm

TABLES

Table 1. Designation and sequences of the synthetic peptides used in this study.

Peptide designation	Sequence
PLP$_{178-191}$ (native sequence)	NTWTTCQSIA**F**PSK
A188	NTWTTCQSIA**A**PSK
S-palmA188	NTWTT**C(Palm)**QSIA**A**PSK
A188-Biotin	NTWTTCQSIA**A**PS**K(Biot)**
S-palmA188-Biotin	NTWTT**C(Palm)**QSIA**A**PS**K(Biot)**
PLP$_{139-151}$ (native sequence)	HCLGK**W**LGHPDKF
Q144	HCLGK**Q**LGHPDFK
S-palmQ144	H**C(Palm)**GK**Q**LGHPDFK

Table 2. The effect of A188 and S-palmA188 on induction of EAE with PLP178-191 and of Q144 and S-palmQ144 on induction of EAE with PLP139-151 in co-immunization experiments at different ratio

Immunized with: (molar ratio)	Incidence	Day of onset[a] (mean ± SE)	Average scores[b] (mean ± SE)	Severity score[c] (mean ± SE)	Disease duration[d] (mean ± SE)
PLP178-191	8/8	11.3 ± 0.5	2.8 ± 0.3	2.8 ± 0.3	6.4 ± 1.1
A188 alone	0/4	-	0	0	0
S-palmA188	0/4	-	0	0	0
PLP178-191 + A188 (1:5)	5/12*	11.5 ± 0.4	1.0 ± 0.4**	2.8 ± 0.4	1.8 ± 0.9**
PLP178-191 + A188 (1:1)	3/4	11.3 ± 0.7	1.9 ± 0.8	2.5 ± 0.9	3.3 ± 1.5
PLP178-191 + S-palmA188 (1:1)	0/8*	-†	0***	0†	0***
PLP178-191 + S-palmA188 (1:0.2)	3/7	14.7 ± 0.9*	0.5 ± 0.3**	1.2 ± 0.4	0.7 ± 0.4**
PLP178-191 + S-palmA188 (1:0.1)	3/8	11.7 ± 0.2	0.9 ± 0.4**	2.3 ± 0.2	2.5 ± 1.5*
PLP139-151	4/4	11.5 ± 0.9	4.3 ± 0.3	4.3 ± 0.3	8.3 ± 0.9
PLP139-151 + Q144 (1:6)	3/4	11.3 ± 0.6	2.1 ± 0.7*	2.8 ± 0.4*	4.3 ± 1.5
PLP139-151 + S-palmQ144 (1:6)	0/4*	-†	0***	0†	0***
PLP139-151 + S-palmQ144 (1:1)	1/3	21†	0.7 ± 0.7**	2†	1.3 ± 1.3**

[a] Average of the day post immunisation that first clinical signs were recorded in the mice that developed EAE

[b] Average of the scores of all mice

[c] Average of the scores of mice that developed EAE;

[d] Average of the number of consecutive days an EAE score was recorded

* $P < 0.05$; ** $P < 0.01$; and ***$P < 0.001$ compared to mice immunized with $PLP_{178-191}$ or $PLP_{139-151}$ alone

[†]Statistical analyses could not be done for these groups due to <3 mice/group with EAE

Table 3. Assessment of proliferation of antigen-primed CD4$^+$ cells, as determined by CFSE and anti-CD4 antibody staining and flow cytometry

Antigen-primed LNC	Stimulating Antigen[¶]	% dividing CD4$^+$ cells (Mean ± SE) [†]	Cell division index
A188	None	1.1 ± 0.2	
	A188	5.2 ± 1.5*	**4.6**
	PLP178-191	1.4 ± 0.3	1.2
	PLP139-151	1.4 ± 0.3	1.2
S-palmA188	None	0.9 ± 0.2	
	A188	10.7 ± 2.6**	**12.2**
	PLP178-191	1.6 ± 0.45	1.8
	PLP139-151	2.6 ± 1.3	2.9
PLP178-191	None	1.5 ± 0.5	
	A188	2.5 ± 0.8	1.7
	PLP178-191	21.4 ± 8.3*	**14.3**
	PLP139-151	2.9 ± 1.3	1.9

*P<0.05; **P<0.01 compared to no antigen control of LNC primed with the same antigen

[¶] ConA was also used as a positive control in all assays (data not shown)

[†]Results are the mean ± SE of 3 replicate experiments

Figure 1.

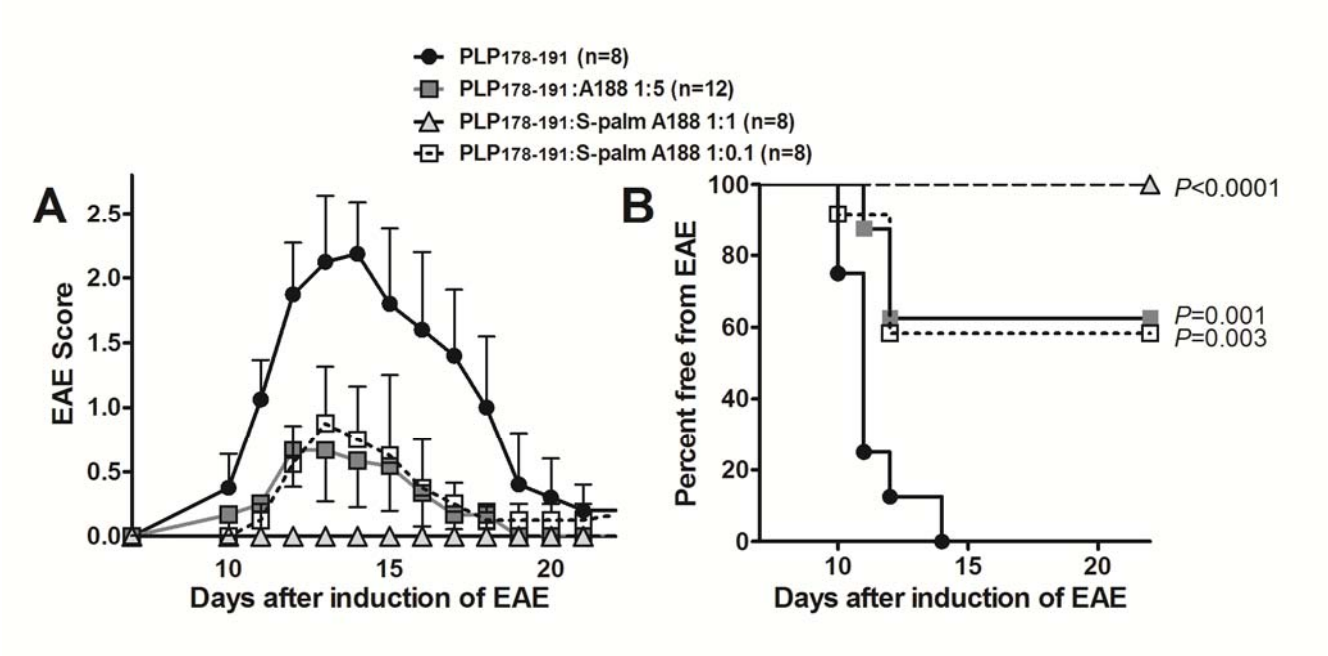

PLP178-191 (n=8)
PLP178-191:A188 1:5 (n=12)
PLP178-191:S-palm A188 1:1 (n=8)
PLP178-191:S-palm A188 1:0.1 (n=8)
A
EAE Score
Days after induction of EAE
B
Percent free from EAE
Days after induction of EAE
P<0.0001
P=0.001
P=0.003

Figure 2.

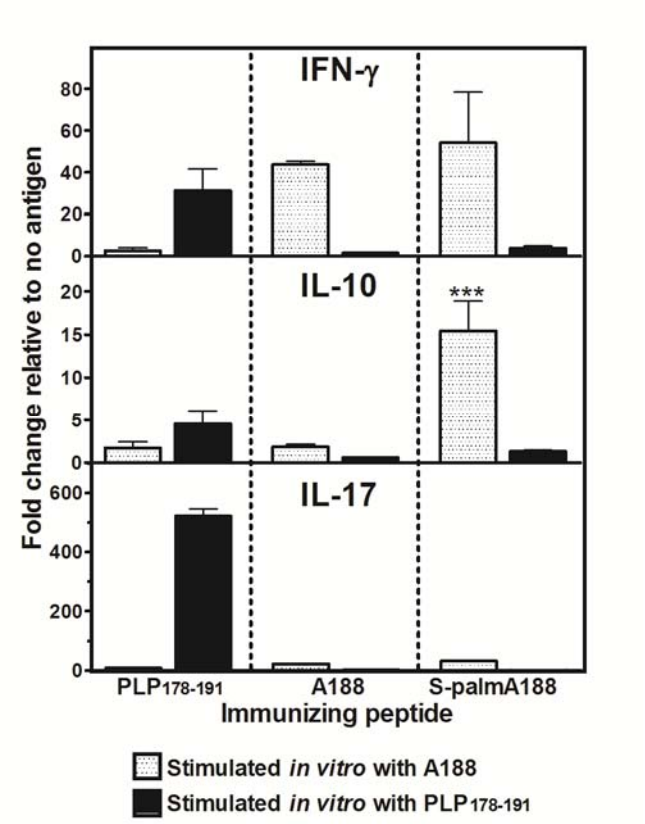

IFN-γ
IL-10
IL-17
***
Fold change relative to no antigen
0
20
40
60
80
0
5
10
15
20
0
200
400
600
PLP178-191
A188
S-palmA188
Immunizing peptide
Stimulated in vitro with A188
Stimulated in vitro with PLP178-191

Figure 3

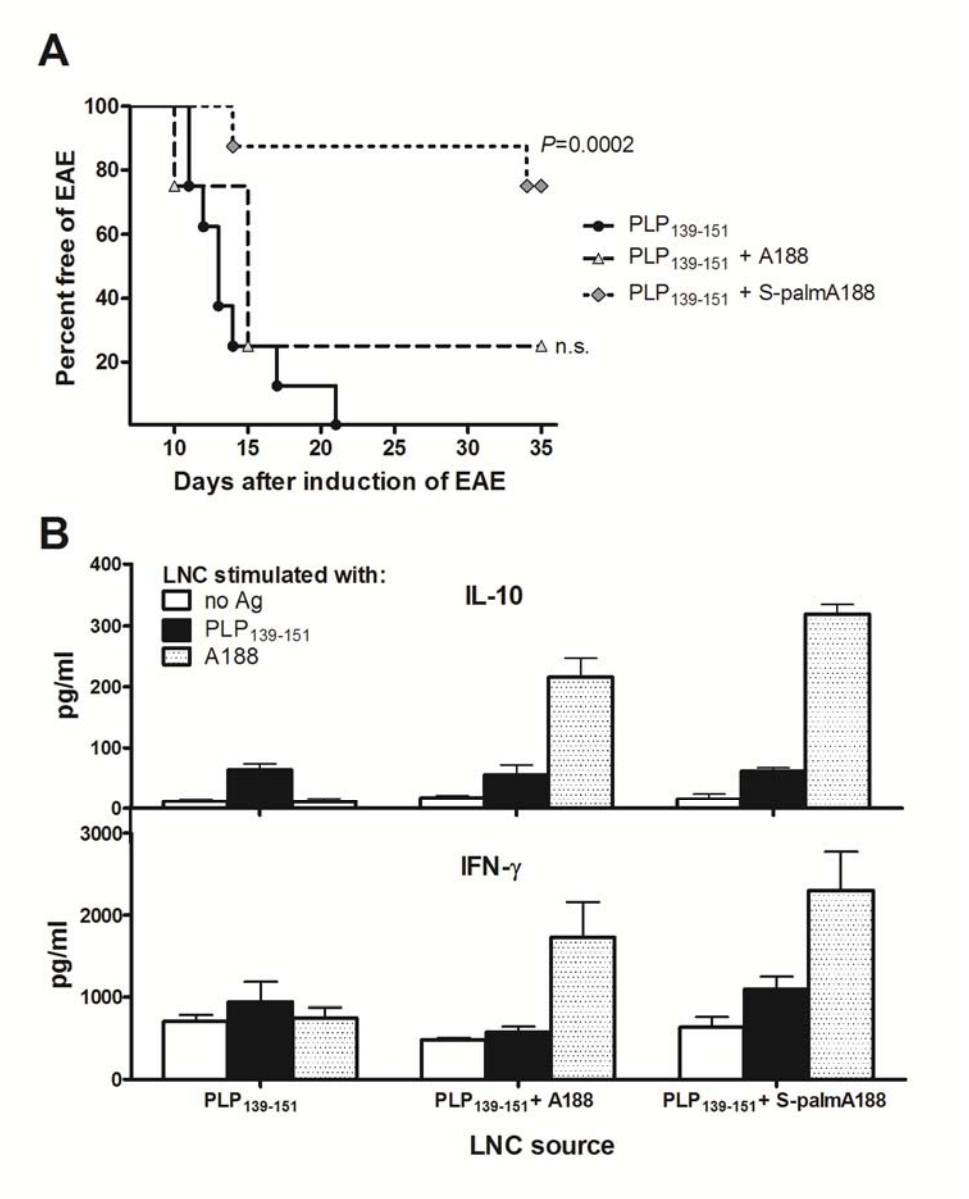

A
Percent free of EAE
Days after induction of EAE
P=0.0002
n.s.
PLP139-151
PLP139-151 + A188
PLP139-151 + S-palmA188
B
LNC stimulated with:
no Ag
PLP139-151
A188
IL-10
IFN-γ
pg/ml
PLP139-151
PLP139-151+ A188
PLP139-151+ S-palmA188
LNC source

Figure 4

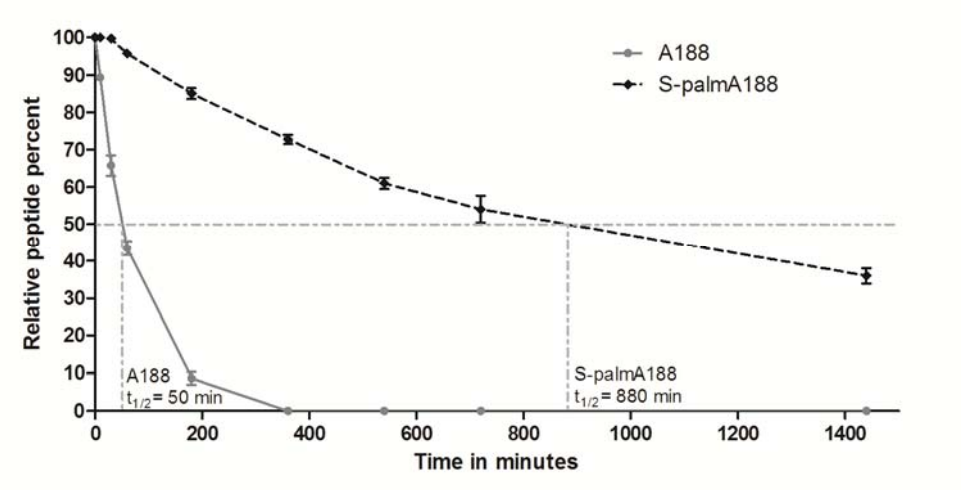
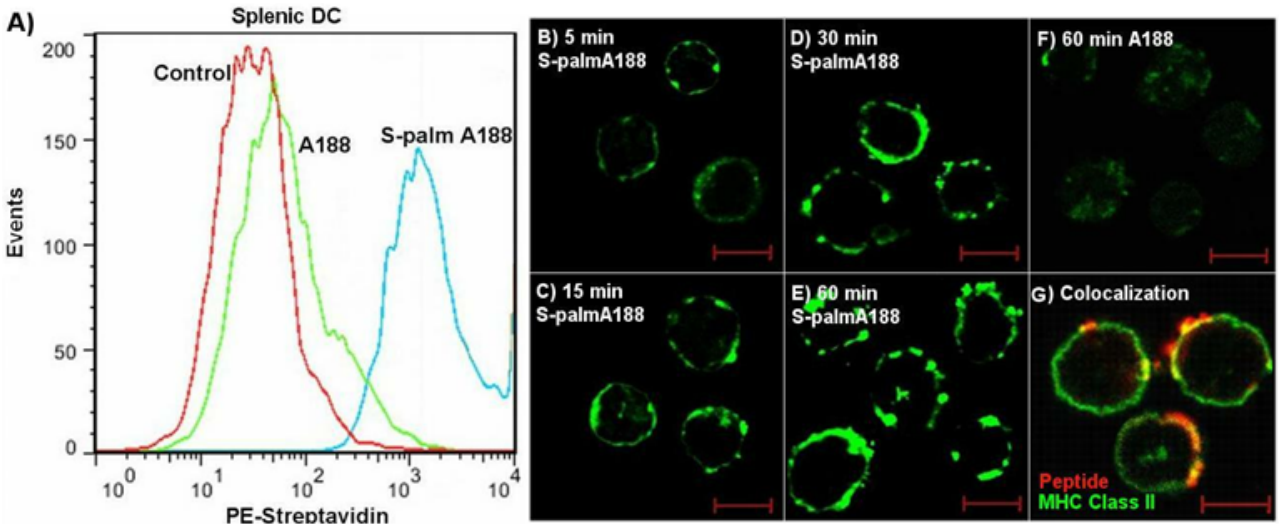

A188
S-palmA188
Relative peptide percent
Time in minutes
A188
t1/2= 50 min
S-palmA188
t1/2= 880 min
0
10
20
30
40
50
60
70
80
90
100
0
200
400
600
800
1000
1200
1400

Figure 5


A)
Splenic DC
Control
A188
S-palm A188
Events
PE-Streptavidin
B) 5 min
S-palmA188
D) 30 min
S-palmA188
F) 60 min A188
C) 15 min
S-palmA188
E) 60 min
S-palmA188
G) Colocalization
Peptide
MHC Class II