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Route of Uptake of Palmitoylated Encephalitogenic Peptides of Myelin Proteolipid Protein by Antigen-Presenting Cells: Importance of the Type of Bond between Lipid Chain and Peptide and Relevance to Autoimmunity[1]

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Previously, we have shown that thiopalmitoylation of peptides of myelin proteolipid protein, as occurs naturally in vivo, increases their ability to induce experimental autoimmune encephalomyelitis, the animal model of multiple sclerosis, and skews the autoimmune response toward a $CD4^+$-mediated response. In contrast, the same peptide, when synthesized with a stable amide bond between peptide and lipid, inhibits experimental autoimmune encephalomyelitis and skews the response toward a $CD8^+$ response. The aim of the current study was to determine the mechanisms responsible for these observations. We show that proteolipid protein lipopeptides, when synthesized with a thioester bond between the lipid and the peptide, are taken up into APCs via an actin-independent endocytic route, the thioester bond is cleaved in the endosome, and the peptide is subsequently displayed on the surface of the APC in the context of MHC class II. The same peptide, when synthesized with the lipid attached via a stable amide bond, rapidly enters into the cytoplasm of the APC and forms micelles; however, the bond between peptide and lipid is not cleaved, and the micelles travel via the endoplasmic reticulum to complex with MHC class I. These findings have implications for vaccine development and for the development of MHC class II-restricted autoimmune diseases, as many human autoantigens thus far identified are thioacylated. *The Journal of Immunology,* **2008, 180: 1398–1404.**

Thioacylation (i.e., the covalent attachment of long-chain fatty acids, particularly palmitic acid, via thioester linkages to cysteine residues in the polypeptide backbone) is a common post-translational modification of proteins that has been implicated in the process of protein trafficking between organelles and in the segregation or clustering of proteins in membrane compartments (1). One thioacylated protein of interest in autoimmunity is myelin proteolipid protein (PLP),[4] the most abundant protein of CNS myelin and a potential autoantigen in multiple sclerosis (MS), which is thioacylated at six sites (2). PLP thioacylation is thought to play an important role in the normal functioning of PLP and in myelin stability, (3) and interestingly, it has been shown that the amount of fatty acid attached to PLP increases during the process of demyelination (4). We have been interested in the role that thiopalmitoylated PLP fragments released during the process of demyelination might play in the expansion of autoimmune responses and enhancement of disease chronicity.

PLP is a potent immunogen in mice and induces experimental autoimmune encephalomyelitis (EAE), an animal model for MS (5–7). Two of the thioacylation sites of PLP (Cys^{108} and Cys^{140}) are within the epitopes of PLP that are encephalitogenic in SJL/J mice, namely $PLP_{104-117}$ and $PLP_{139-151}$ (5, 8). We have previously shown that, in comparison with nonacylated peptides, thiopalmitoylated-$PLP_{104-117}$ and -$PLP_{139-151}$ (S-palm peptides) induced greater $CD4^+$ T cell and Ab responses and enhanced the development and chronicity of EAE (9). Our previous work also suggested that the lability of the thioester bond between the peptide and fatty acid was important for induction of $CD4^+$ T cells, as PLP lipopeptides synthesized with the fatty acid attached via an amide linkage at the N terminus (N-palm peptides) induced a T cell response with a decreased CD4/CD8 ratio, failed to induce T cell proliferation, and were not encephalitogenic (9).

To understand how the S-palm and N-palm PLP peptides could induce such different outcomes, and to further our understanding of the role that thiopalmitoylated lipopeptides might play during the demyelination process in EAE and MS, we have used $PLP_{139-151}$ lipopeptides, in which both the peptide and palmitic acid are labeled, to investigate how they are taken up and processed in APC. We show that the stability of the bond between peptide and lipid affects the route of the peptide through the APC and how it will eventually be presented to the immune system. These findings have implications for development of MHC class II-restricted

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[4] Abbreviations used in this paper: PLP, proteolipid protein; MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; PC, peritoneal cell; ER, endoplasmic reticulum; AOTF, acousto-optic tunable filter.

Table I. *Sequences of the peptides used in this study*

Peptide Designation	Sequences	Molecular Mass
$PLP_{139-151}$	H_2N-HCLGKWLGHPDKF-COOH	1537.8
$PLP_{139-151}$ Biot	H_2N-HCLGKWLGHPDK(Biot)F-COOH	1764.1
S-palm $PLP_{139-151}$	H_2N-HC(Palm)LGKWLGHPDKF-COOH	1776.2
S-palm $PLP_{139-151}$ Biot	H_2N-HC(Palm)LGKWLGHPDK(Biot)F-COOH	2002.5
N-palm $PLP_{139-151}$	Palm-HCLGKWLGHPDKF-COOH	1776.2
N-palm $PLP_{139-151}$ Biot	Palm-HCLGKWLGHPDK(Biot)F-COOH	2002.5
S-P12 $PLP_{139-151}$ Biot	H_2N-HC(P12)LGKWLGHPDK(Biot)F-COOH	2147.6
N-P12 $PLP_{139-151}$ Biot	P12-HCLGKWLGHPDK(Biot)F-COOH	2147.6

autoimmune diseases and also potentially for the use of lipopeptides as vaccines.

Materials and Methods

Macrophages

Peritoneal cells (PC) from normal 8–12-wk old female SJL/J mice (from Charles River Laboratories or from the Animal Resources Centre) were used as a source of macrophages. Mice were maintained and used in accordance with the University of Queensland and Université Louis Pasteur ethical guidelines for use of experimental animals, and the experimental protocol was approved by their Animal Experimentation Ethics Committees. Mice were euthanized by CO_2 inhalation, and PC were obtained by i.p. injection of 7 ml of PBS into the mice, followed by recovery of the fluid containing PC. Approximately 40% of these cells were $F4/80^+$ macrophages, as determined by flow cytometry analysis.

Peptide synthesis

Labeled and non-labeled $PLP_{139-151}$ lipopeptides were synthesized by solid-phase synthesis using a Fmoc/tBu strategy, as previously described (10, 11). The side chain of the cysteine 140 residue to be modified by palmitoylation was protected with a 4-methoxytrityl group allowing selective deprotection by 2% trifluoroacetic acid in dichloromethane, followed by on-resin acylation with palmitic acid and benzotriazol-yl-1-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP). The N-Palm peptide was obtained by deprotection of the His^{139} (Fmoc) with 20% piperidine in dimethylformamide, followed by coupling activated palmitic acid on the N terminus residue. The Lys^{150} (Dde) was selectively deprotected with a solution of 2% hydrazine monohydrate in dimethylformamide, followed by the biotinylation of the N^{ε}-Lys^{150} with biotin activated by BOP. 12-(1-Pyrenyl) dodecanoic acid (P12) was synthesized as described by Katušin-Ražem (12) with slight modifications to the conditions to form the bromide phosphonium salt and the hydrogenation reaction. Pure P12 was obtained in four steps in 52% yield. The S-P12 and N-P12 peptides were obtained in the same way as described above for the S-palm and N-palm peptides, with P12 substituted for palmitic acid. After cleavage from the resin, the crude peptides were lyophilized and purified by C4 RP-HPLC. The purity of the peptides was ≥95%, and their identities were confirmed by molecular mass determination on a MALDI-TOF spectrometer. The sequences of the peptides are shown in Table I.

Antibodies

PE-labeled F4/80 (Serotec) was used at 1/10 dilution to label macrophages. Mannose-6-phosphate receptor (2G11), endoplasmic reticulum (ER) (protein disulfide isomerase), and CD107a (LAMP-1) Abs (all from Abcam plc) were used at 1/100 dilution to label endosomes, ER, and lysosomes, respectively. Clone KH49 (anti-H-$2K^s$ (MHC class I)) and 7-16.17 (anti I-$A^{p,s}$ (MHC class II)) were obtained from BD Biosciences and were both used at 1/100 dilution. In addition, FITC-labeled streptavidin (used at 1/200) was obtained from Serotec, and Texas Red-labeled rabbit polyclonal Ab to mouse IgG (H&L chains) was obtained from Abcam and used at 1/400 dilution. Anti-CD3-PE, anti-CD4-PerCP, and anti-CD8-PerCP were obtained from BD Pharmingen and were used at a concentration of 1 μg/ml.

Flow cytometry

PC were washed and incubated with peptide for various times at 37 or 5°C. At the end of the incubation, they were fixed in freshly prepared 4% formaldehyde in PBS for 15 min at room temperature. After two washes with PBS, PC were permeabilized with 0.05% digitonin for 5 min at room temperature, washed twice more with PBS containing 1% FCS and 0.01% sodium azide, and double stained with PE-labeled F4/80 Ab to detect macrophages and FITC-streptavidin to detect the biotinylated peptide. After washing in PBS containing 1% FCS and 0.01% sodium azide, the cells were analyzed by flow cytometry using a FacsCalibur system (BD Biosciences). Samples were gated on the $F4/80^+$ population, and the mean fluorescence intensity of staining with FITC-streptavidin was determined.

Confocal microscopy

Macrophages were isolated from PC suspensions by adhesion to glass coverslips. The macrophages were then incubated with 100 μM peptide for between 1 min and 24 h at 37 or 5°C. After incubation, cells were fixed with 4% formaldehyde in PBS for 15 min at room temperature and permeabilized with 0.05% digitonin in PBS for 5 min at room temperature or 0.2% Triton X-100 in PBS for 20 min at room temperature. In colocalization experiments not involving P12, cells were then incubated with murine Abs against endosomes, lysosomes, ER, or MHC class I or II molecules for 1–3 h at 37°C. After three washes in PBS, cells were incubated with streptavidin-Alexa 488 (1/400 dilution in PBS) or streptavidin-Cy3 (1/450 dilution in PBS) (both from Molecular Probes) and Texas Red-labeled rabbit anti-mouse IgG or IgM for 30 min in the dark at room temperature. After washing with PBS, coverslips were mounted in Aquapolymount medium. Immunofluorescence staining was monitored with a laser scanning microscope (LSM 510; Carl Zeiss Laboratories) equipped with a Plan-Apochromat 63× oil DIC immersion lens (numerical aperture 1.4). Alexa 488 emission was excited using the 488-nm ray of the argon laser, whereas Texas Red was excited using the 543-nm line of the helium/neon laser. Emission signals of Alexa 488 and Texas Red were filtered with a LP 505–530 and a LP 560 filter, respectively. Quantification of colocalization was performed on cells from 10 to 12 fields (at least 100 cells) with the colocalization module of the Zeiss LSM Image Browser software. Statistical analyses were performed using GraphPad Prism 4 (GraphPad). Unpaired two-tailed *t* tests were used to compare colocalization between cells treated with S-palm and N-palm peptides.

For the experiments using P12 peptides, streptavidin-Cy3 (1/450 dilution in PBS) (Molecular Probes) was used instead of streptavidin-Alexa 488, and immunofluorescence staining was monitored with an inverted laser scanning microscope (DMIRBE, SP2, Leica Microsystems) equipped with a Plan-Apochromat 63× oil immersion lens (numerical aperture 1.4). Cy3 emission was excited using the 568-nm ray of the krypton laser, whereas P12 was excited at 351/364-nm using the 50mW UV ray of the argon laser, and at 488-nm with the argon laser. Emission signals from Cy3 were recorded using Leica's spectral detection system between 580 and 700 nm and acousto-optic tunable filter (AOTF) 25%, and emission signals from P12 were recorded with Leica's spectral detection system between 498 and 529 nm (excimer form) and AOTF 30% or between 400 and 500 nm without AOTF (monomer form).

Cytochalasin D treatment

To study the effect of cytochalasin D upon peptide uptake, PC were pretreated with 20 μM of cytochalasin D (from 5 mg/ml stock solution in DMSO diluted in DMEM) for 30 min at 37°C. Control cells were mock treated with medium supplemented with DMSO only at the concentration used in cytochalasin D-supplemented media. Following this treatment, the medium was aspirated and cells were incubated with peptides as described above.

CFSE assay for proliferation of T cells

Pooled lymph node cells were prepared from inguinal lymph nodes from two to three mice injected 8 days earlier with a single s.c. injection of 100 μg of $PLP_{139-151}$ in CFA. Lymph node cells were washed twice in PBS

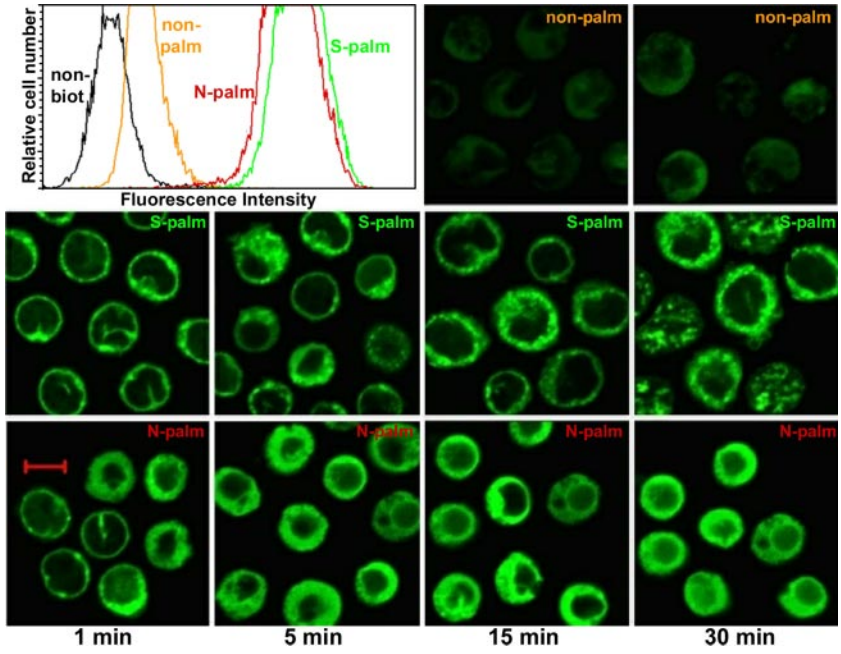

FIGURE 1. Comparison of the uptake of S-palm, N-palm, and nonpalmitoylated $PLP_{139-151}$ by macrophages. Uptake of 100 μM of $PLP_{139-151}$ (non-biot), $PLP_{139-151}$ Cys^{140}-SH Lys^{150}-Biot (nonpalm), $PLP_{139-151}$ Cys^{140}-Palm Lys^{150}-Biot (S-palm), or $PLP_{139-151}$ His^{139}-Palm Lys^{150}-Biot (N-palm) peptides into $F4/80^+$ macrophages was monitored by flow cytometry (at 15 min) and by confocal microscopy after 1-, 5-, 15-, and 30-min incubation. The efficiency of uptake of both S-palm and N-palm peptides is much greater than nonpalm peptide, but the S-palm peptide is taken up into discrete vesicular compartments, whereas the N-palm peptide is homogeneously distributed throughout the cytoplasm of the cells. Bar = 5 μm.

and labeled with 0.2 μM CFSE (Invitrogen Life Technologies), then cultured in vitro in phenol red-free RPMI 1640 medium supplemented with 10% FCS in the presence or absence of Ag for 5 days. After that time, cells were harvested and stained with anti-CD3-PE and anti-CD4-PerCP or anti-CD8-PerCP. Cells were gated on $CD3^+$ lymphocytes, and the numbers of $CD4^+$ or $CD8^+$ cells proliferating, and thereby showing decreased levels of CFSE staining, were determined. The results are expressed as the percentage of cells dividing (number of $CD4/CD8^+$ T cells with decreased levels of CFSE staining divided by total number of $CD4/CD8^+$ T cells).

Results

S-palm and N-palm $PLP_{139-151}$ show similar kinetics and efficiency of uptake into peritoneal macrophages

In a previous study, we have shown that the entry of the S-palm peptide into an APC is more rapid and efficient than uptake of nonpalmitoylated peptides (11). In the current study, we first compared the uptake of 100 μM of biotinylated S-palm $PLP_{139-151}$, N-palm $PLP_{139-151}$, or nonpalmitoylated $PLP_{139-151}$ into SJL/J peritoneal macrophages. Macrophages were used in this study, as they are the predominant APC type in the CNS during EAE and MS (13), and it has been shown that they are very efficient at taking up myelin breakdown products, transporting them to the deep cervical lymph nodes, and presenting these Ags to effector T cells (14–16). They can also present Ag to naive T cells, but to a lesser extent than dendritic cells (16). However, due to their abundance in the lesion during EAE or MS, macrophages are the most likely cells to be taking up and presenting thiopalmitoylated lipopeptides during EAE or MS and are, therefore, used as APC in the current study. Within 1 min, >90% of the macrophages incubated with either S-palm or N-palm peptide were strongly labeled, and this increased to 99% for these peptides after 15 min, as shown by flow cytometric analysis of the cells (Fig. 1, *upper left panel*). In contrast, the nonpalmitoylated peptide was taken up only very slowly and to a much lesser extent by macrophages. Thus, the efficiency of uptake of both the S-palm and N-palm peptides is much greater than for nonpalmitoylated peptide. The uptake of the nonpalmitoylated and palmitoylated peptides into dendritic cells purified from SJL/J splenocytes was almost identical to that seen in macrophages (results not shown).

S-palm and N-palm $PLP_{139-151}$ are taken up into different subcellular compartments and presented by different MHC molecules

The route of internalization of the S-palm and N-palm peptides into macrophages at 37°C was initially determined by following the biotinylated peptides using fluorescence confocal microscopy over the course of 30 min (Fig. 1). After 1 min, S-palm peptide was located primarily at the cellular membrane and after 5 and 15 min, a punctate fluorescence pattern was observed, indicative of localization of the translocated peptide in discrete vesicular compartments in the cytoplasm and suggestive of an endocytic pathway of internalization. By 30 min, the S-palm peptide was fully vesicular (Fig. 1). In contrast, after 1 min, the N-palm peptide was located not only at the cellular membrane, but, in addition, homogeneous cytosolic staining could be observed in some cells. By 5 min, the fluorescence pattern was homogenous throughout the cytoplasm of all cells incubated with N-palm peptide. After 15 min, the fluorescence was homogeneous and peripheral to the nucleus, suggestive of ER localization (Fig. 1). The lipid moiety induced internalization of the peptides, since the control nonpalmitoylated biotinylated peptide was only faintly visualized inside the cells, even after 30 min of incubation (Fig. 1). The rate of internalization of both S-palm and N-palm peptides was slowed at 5°C and was not inhibited by treatment of the cells with cytochalasin D (which blocks actin polymerization), suggesting an active actin-independent endocytic mechanism.

Confocal microscopy, using anti-mannose-6-phosphate receptor (2G11) and anti-LAMP-1 (LAMP-1) as markers for late endosomes and lysosomes, respectively, was employed to determine and quantify the colocalization of the S-palm and N-palm peptides with specific intracellular organelles within the macrophages at 1, 15, 30, and 60 min of incubation. After 1 min, much of the S-palm peptide was already associated with endosomes close to the cell surface (Fig. 2*A*). By 30 min, however, the amount of S-palm peptide associated with late endosomes had started to decrease, and there was an increase in the amount of S-palm peptide associated with lysosomes (Fig. 2*B*). In contrast, N-palm peptide did

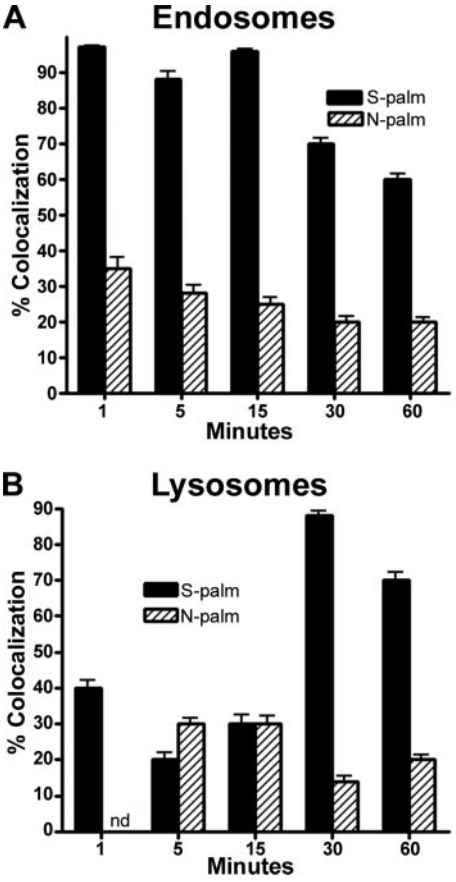

FIGURE 2. S-palm peptide colocalizes strongly with endosomes and lysosomes. *A*, Macrophages were incubated with biotinylated S-palm or N-palm $PLP_{139-151}$ for 1, 5, 15, 30, or 60 min and then stained to detect endosomes or peptide. The percentage of endosomes that colocalized with peptide was determined using the colocalization module of the Zeiss LSM Image Browser software. S-palm peptide rapidly colocalized with endosomes. N-palm peptide did not colocalize strongly with endosomes in most cells. Bars represent the percentage colocalization (mean ± SE) in at least 100 cells. *B*, The percentage of lysosomes that colocalized with peptide was determined as described for endosomes. N-palm peptide did not colocalize strongly with lysosomes in most cells, and there was no time-dependent increase of this localization. nd = not done.

not colocalize strongly with endosomes or lysosomes in the majority of cells, and there was no time-dependent increase of this localization. We therefore investigated whether N-palm peptide colocalized with ER, using anti-protein disulfide isomerase Abs as an ER marker. After 30 and 60 min of incubation, colocalization of the N-palm peptide with ER was increased in comparison to colocalization of S-palm peptide with ER (Fig. 3), indicating that N-palm peptide follows a more cytoplasmic pathway.

Since S-palm peptide generally followed an endocytic pathway and N-palm peptide associated with the ER, we next used confocal microscopy and Abs against MHC class I and II molecules to determine whether the different lipopeptides associated with particular MHC molecules. After 1 h of incubation, the colocalization of S-palm peptide with MHC class II on the surface and within subcellular compartments of the macrophages was significantly greater ($p < 0.0001$) than its colocalization with MHC class I (Fig. 4*A*). In contrast, N-palm peptide colocalized with MHC class I throughout the cytoplasm and on the cell surface, but not with MHC class II ($p < 0.001$) (Fig. 4*B*). Interestingly, macrophages incubated with S-palm peptide showed increased levels of expression of MHC class II, but not MHC class I, as indicated by stronger

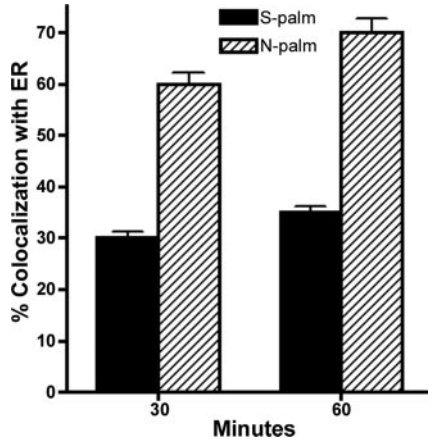

FIGURE 3. Macrophages were incubated with biotinylated peptide for 30 or 60 min and then stained to detect ER or peptide. The percentage of ER colocalized with peptide was determined as in Fig. 2. Colocalization of N-palm peptide and ER increased after 30 min of incubation, compared with colocalization of S-palm peptide with ER.

staining with the specific Abs, whereas macrophages incubated with N-palm peptide showed increased levels of expression of MHC class I, but not MHC class II.

When does the fatty acid dissociate from the peptide?

To determine whether and when the fatty acid and peptide dissociate within the cell, we synthesized biotinylated thioacylated peptides in which the palmitic acid was replaced by the fluorescent analog P12 to give S-P12 and N-P12 peptides. P12 has previously been shown to be a good analog for palmitic acid in cell biology studies (17). Furthermore, pyrene lipid probes have interesting fluorescent properties, as they are able to form excimers (dimers in which one excited pyrene reacts with another in the fundamental state), with red-shifted fluorescence emission, depending on their

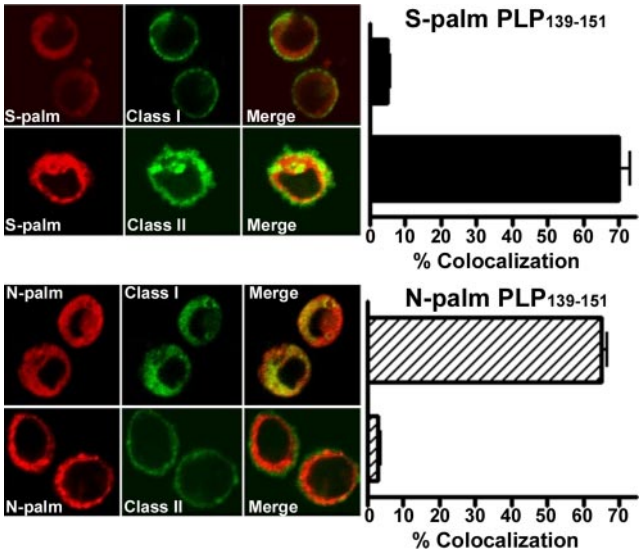

FIGURE 4. Colocalization of palmitoylated $PLP_{139-151}$ with MHC molecules. Macrophages were incubated with biotinylated S-palm or N-palm peptide for 1 h and then stained to detect MHC class I or II and peptide. S-palm peptide colocalized strongly with MHC class II, but there was only a small percentage of S-palm peptide that colocalized with MHC class I ($p < 0.0001$). In contrast, N-palm peptide colocalized strongly with MHC class I and to a much lesser degree with MHC class II ($p < 0.0001$).

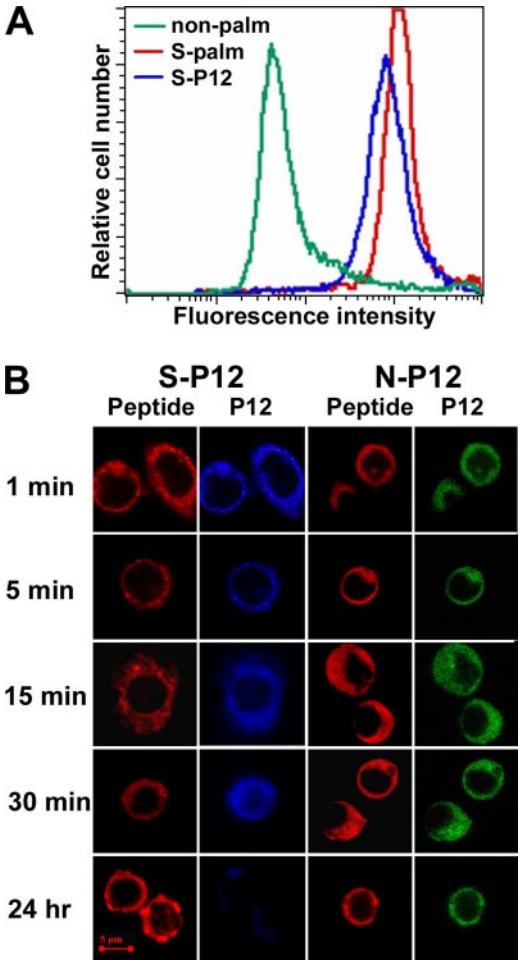

FIGURE 5. Macrophages were incubated with biotinylated P-12 peptides to determine the fate of the peptide and lipid portions of the lipopeptides. *A*, There were no differences in the uptake of the S-P12 peptide compared with S-palm peptide after 1 h incubation. *B*, P12 was rapidly cleaved from the peptide when it was attached via a thioester linkage (S-P12), as indicated by the differences in the staining pattern of the peptide and P12 portion of the molecule. In addition, the P12 remained in a monomeric form over the course of 24 h, as indicated by the blue fluorescence. In contrast, N-P12 peptide did not dissociate but formed excimers or remained in a micellar form over the course of 24 h, as indicated by the green fluorescence.

local concentration and proximity; thus, the excited monomers exhibit a blue fluorescence, whereas excited dimers exhibit a green fluorescence (18). Dimers in a supramolecular association, such as in micelles, also exhibit a green fluorescence. Thus, in our study, the peptide part of the lipopeptide and the fatty acid chain could be followed independently and the fluorescent color, exhibited by the P12, gave information about the local concentration of the palmitic acid chain.

There were no major differences in the uptake of either P12 peptide by peritoneal macrophages, compared with the uptake of the corresponding S-palm (Fig. 5*A*) or N-palm (not shown) peptide. In colocalization studies at 37°C in macrophages using S-P12 peptides (Fig. 5*B*), the peptide and lipid portions were colocalized up until 5 min. After 15 min, the biotinylated peptide part of the molecule followed the endocytic pathway described above for the S-palm peptide, but the P12 was distributed throughout the cell. These results show that once the lipopeptide is inside the cell, the P12, attached via a thioester linkage, is rapidly dissociated from the peptide. After 24 h, little residual P12 fluorescence could be

Table II. *Proliferation of $CD4^+$ and $CD8^+$ T cells from mice immunized with $PLP_{139-151}$ in response to in vitro activation with nonpalmitoylated, S-palm, or N-palm peptide*[a]

Antigen	% $CD4^+$ T Cells Dividing	% $CD8^+$ T Cells Dividing
No antigen	1.20 ± 0.17	1.34 ± 0.52
$PLP_{139-151}$	4.54 ± 2.09	2.48 ± 0.56
S-palm $PLP_{139-151}$	5.55 ± 1.84	1.14 ± 0.19
N-palm $PLP_{139-151}$	0.98 ± 0.02	1.50 ± 0.34
Con A	18.24 ± 5.02	21.03 ± 3.69

[a] The results show the percentage of CFSE-labeled T cells (gated on $CD3^+$ cells) (±SD from three replicate experiments) that divided after 7 days in culture. S-palm $PLP_{139-151}$ induced proliferation of $CD4^+$ T cells but not $CD8^+$ T cells.

detected within the macrophages. At all time points, the P12 chain of the S-P12 peptide fluoresced blue, with no detectable green fluorescence, indicating that it remained in a monomeric form, probably due to its dilution within subcellular compartments or membranes.

In contrast, when the P12 was attached to the peptide via a N-terminal amide linkage, the peptide and lipid chain showed an identical distribution at all time points up to 24 h (Fig. 5*B*), and the P12 exhibited a green fluorescence at all times, indicating the presence of excimers and probably reflecting the association of the N-P12 peptide molecules in micelles, due to the amphipathic nature of the lipopeptide. Thus, the linkage between peptide and lipid in the N-P12 peptide is extremely stable.

What is the fidelity of the Ag processing pathway?

To ascertain the fidelity of the processing pathway in live cells, mice were immunized with $PLP_{139-151}$, and lymph nodes cells from these mice were collected 8 days later, stained with CFSE, and then cultured for 5 days in the presence or absence of nonacylated $PLP_{139-151}$, S-palm $PLP_{139-151}$, or N-palm $PLP_{139-151}$. The results (Table II) show the percentage of $CD4^+$ or $CD8^+$ T cells dividing in response to each of these peptides or to the mitogen Con A. $CD4^+$ T cells from $PLP_{139-151}$-immunized mice responded more strongly to the S-palm peptide than to the nonacylated peptide, and the N-palm peptide did not induce any response in the $CD4^+$ T cells. $CD8^+$ T cells, in contrast, did not proliferate in response to either the S-palm or N-palm peptide. These results are in agreement with what we have previously described (9) and with our findings in the macrophages. They show that the majority of the S-palm peptide enters the MHC class II presentation pathway and is presented to $CD4^+$ T cells. As suggested from the results with the P-12 peptide, the N-palm peptide is in a multimeric form, and even though it is colocalized with MHC class I, the physical conformation prevents it from interacting effectively with the $CD8^+$ T cells. In contrast, the nonacylated $PLP_{139-151}$ peptide can enter both the class I and II pathways and stimulate both $CD4^+$ and $CD8^+$ T cells.

Discussion

This study demonstrates that induction of immune responses with encephalitogenic PLP lipopeptides can result in different outcomes, depending on the way in which the fatty acid chain is attached to the peptide backbone. We show that when fatty acid is attached via a labile thioester bond, as occurs naturally in the body, it predisposes toward uptake of peptides into the endosomal/lysosomal pathway and subsequent presentation of these peptides in the context of MHC class II. We believe that this finding may be particularly relevant in the development of class II-restricted autoimmune disease. In this study, we have used macrophages as

APC, since they are the most abundant APC type present in the EAE or MS lesion at the time of demyelination, when thiopalmitoylated peptides are likely to be released and taken up by APC (13). In addition, previous studies have shown that myelin-debris-laden macrophages from the CNS can travel to the CNS-draining lymph nodes and present myelin Ag to T cells (14–16).

The majority of lipopeptides that have been investigated for their potential as enhancers of vaccine activity have been synthesized with stable bonds between the lipid moiety and the N terminus of the peptide, and the types of responses that have been shown to be induced in these cases have been predominantly $CD8^+$ T cell responses (19–22). In contrast, Beekman et al. (23, 24) showed that S-palm peptide vaccines induced good Ab responses. We have previously found that S-palm $PLP_{104-117}$ and $PLP_{139-151}$ induce strong $CD4^+$ T cell-mediated immune responses and are encephalitogenic in mice (9). In accordance with the previous studies using N terminus-modified peptides, we found that N-palm $PLP_{139-151}$ skewed the T cell response toward a decreased CD4/CD8 ratio and failed to induce EAE (9). In the current paper, we have shown that the underlying reason for these different outcomes is differences in the stability of the bond between the peptide and lipid in the environment inside the APC and the pathway the peptide follows within the APC.

The presence of a palmitic chain attached to a peptide is sufficient to allow the peptide to cross cellular (but not nuclear) membranes (25). Indeed, transient palmitoylation of bioactive peptides is one mechanism by which they can be transported across membranes. In the case of the peptides used in this study, both S-palm and N-palm peptides will cross the endosomal membrane and enter the endosome. The difference is that the palmitic acid is cleaved from the S-palm peptide in the endosome, preventing the free peptide from subsequently leaving the endosome and, thus, favoring processing of that peptide via the MHC class II presentation pathway. Cleavage of the palmitic chain from the peptide in the S-palm peptide occurred within 15 min of entry of the peptide into the cell, when the majority of the lipopeptide is in the late endosomes. Palmitoyl protein thioesterases, which cleave thioester bonds, localize to late endosomes and lysosomes (26). The free fatty acid can then enter all parts of the cell, whereas the free peptide remains in the vesicles, trafficking via the lysosomes and complexing with MHC class II molecules for eventual presentation on the surface of the APC.

In contrast, the amide bond of the N-palm peptide is stable and resistant to the degradation enzymes within the APC, allowing most of the lipopeptide to rapidly exit the endosomes and gain access to the cytosol in a micellar form. Such an endosome-to-cytosol pathway has already been shown to occur for an N-palm lipopeptide derived from an HIV-1 reverse transcriptase epitope (27), as well as for other lipid-containing compounds, allowing for MHC class I presentation of exogenous N-palm peptides (28). The N-palm micelles then concentrate in the ER and, eventually, the peptide complexes with MHC class I molecules. A small amount of the N-palm peptide can also colocalize with MHC class II (Fig. 4). Our results strongly suggest that the MHC molecules (class I or II) can bind the peptide still conjugated to the lipid, because dissociation of the lipid from the peptide would have resulted in a color shift in the emission spectra in the N-P12 from green to blue, due to dilution of the free lipid in subcellular compartments or membranes. Although the MHC class I binding cleft is restrictive in the size of peptides it can bind (29), Loing et al. (30) have previously suggested that the lipid moiety can remain attached to the peptide part of a lipopeptide and not interfere with MHC class I binding.

We found previously that N-palm PLP peptides were less immunogenic than the corresponding nonpalmitoylated peptides or S-palm peptides (9) and show in the current paper that they are poor inducers of either class I- or II-restricted recall responses (Table II). This is not due to decreased uptake into the macrophages, as we have shown here that they are taken up as well as S-palm peptide and much better than the nonpalmitoylated peptide. However, it could be due to a decreased binding affinity for MHC, as was observed by Andrieu et al. (27) for a HIV-1 reverse transcriptase lipopeptide epitope and/or interference in TCR binding to the MHC-Ag complex, which would be likely in our system where the N-P12 peptide is still in an associated form, as indicated by the green fluorescence in Fig. 5, even when it is colocalized with MHC. Other studies have also shown that N-palmitoylation of peptides decreases the immune response in comparison to the response induced by the same nonpalmitoylated peptide: St. Louis and colleagues (31) showed that N-palmitoylation of normally encephalitogenic epitopes of myelin basic protein rendered the peptide nonimmunogenic both in vitro and in vivo. Furthermore, they showed that an N-palm PLP peptide was able to actively suppress the response to the nonacylated peptide (32). Bueno et al. (33) found that an N-palm peptide of pigeon cytochrome C could interact with a MHC class II-restricted $CD4^+$ T cell clone, but this interaction was not nearly as robust as that occurring with the nonpalmitoylated peptide; indeed N-palmitoylation of the peptide converted it into a weak agonist that could then only induce T cell anergy. A possible explanation for all of these observations with N-palm peptides is that the peptides are forming multimers, as we have found in our system, thus inhibiting formation of the trimolecular complex of MHC/peptide/TCR to some degree.

Of particular interest is the role that thioacylated Ags might play in autoimmunity. In our previous work, we have shown that S-palm PLP peptides enhanced the development and chronicity of EAE and have suggested that naturally occurring S-palm PLP peptides generated as a consequence of myelin breakdown during MS may contribute to the chronic immune attack characteristic of this disease (9, 34). In addition to MS, other common human autoimmune diseases, e.g., insulin-dependent diabetes mellitus and rheumatoid arthritis, have been linked to carriage of particular MHC class II molecules, and several well-characterized autoantigens are known to be thioacylated, e.g., GAD-65 (in insulin-dependent diabetes mellitus), P0 (autoimmune neuritis), erythrocyte band 3 (autoimmune hemolytic anemia), rhodopsin (autoimmune uveoretinitis), and myelin-associated glycoprotein (autoimmune sensory neuropathy) (35–39). We postulate that these Ags may be made more autoantigenic by the presence of the thioacyl chain. Even if these thioacylated proteins are not the primary target of an autoimmune attack, the presence of the thioacyl chain might result in a greater tendency for autoreactivity to spread to them, leading to the development of chronic autoimmune disease.

This work has potential application for development of methods to specifically induce MHC class II-restricted responses. A variety of strategies have been used to enhance immunogenicity and to force Ags into a class I or class II presentation pathway, including linking Ag to the sorting signal of LAMP-1 to get it into an endosomal/lysosomal pathway, linking Ag to bacterial toxins and heat-shock proteins, and the use of chemokine-Ag fusion constructs (40, 41). Thiopalmitoylation of Ags may provide a simpler means to induce MHC class II-restricted responses, with its dual functions of enhancing loading of the Ag into the cell and increasing delivery of the Ag into endosomes and lysosomes.

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Disclosures

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