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Essential role for cholesterol in the delivery of exogenous antigens to the MHC class I-presentation pathway

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Abstract

Cross-presentation, which is crucial for the generation of immunity against virus-infected and tumor cells, requires exogenous antigens to be internalized into antigen-presenting cells (APCs) followed by translocation to the cytosol by unknown mechanisms. One important entry route for such antigens is macropinocytosis. We here describe that cholesterol is essential for cross-presentation of antigens loaded via macropinocytosis into APCs. Modification of antigens by palmitoylation to target antigens to cholesterol-enriched plasma membrane domains resulted in a dramatically increased T cell activation. These results define cholesterol as an essential factor for cross-presentation and suggest that specific modification of antigens to increase their affinity for cholesterol may be utilized to enhance immunity.

Introduction

Generation of an immune response occurs through the activity of the MHC class I and MHC class II molecules, whose function is to present foreign peptides to CD8 and CD4 positive lymphocytes, respectively. MHC class II molecules form a complex with an invariant chain in the endoplasmic reticulum (ER) and are targeted to post-Golgi endosomal/lysosomal organelles, the so-called MHC class II compartments (1–3). Within these organelles, they assemble with peptides derived from exogenous antigens that are internalized via endocytosis and degraded in endosomal/lysosomal organelles (4–7). These intracellularly formed MHC class II–peptide complexes are then transported to the cell surface for presentation to CD4+ (helper) T lymphocytes (8).

MHC class I molecules, in contrast, are synthesized in the ER, assemble with beta 2-microglobulin and remain in the ER until they become loaded with antigenic peptides (9). Antigenic peptides loaded onto MHC class I molecules are derived from antigens as well as infectious agents that reside in the cytosol, such as viral proteins as well as tumor-derived antigens (10, 11). These cytosolic antigens are degraded by proteasome, a cytosolic multi-protease complex, into peptides (12, 13), which are subsequently translocated into the ER in an ATP-dependent manner by the transporter associated with antigen processing (TAP) (14, 15). Assembly of antigenic peptides with the MHC class I/beta 2-microglubulin complex in the ER triggers their transport to the cell surface where they can activate CD8+ (killer) T lymphocytes (16). This dichotomy between the MHC class I and class II pathways ensures the efficient and selective killing of virally infected or tumor cells, while generating a help response in case of bacterial infections (4, 17). In addition, restricting MHC class I presentation to endogenous antigens prevents healthy cells from becoming targets for killing by CD8+ T lymphocytes (18).

An important step in the generation of an immune response is the activation of naive T cells, which occurs through their stimulation by dendritic cells (19–21). Dendritic cells sample peripheral tissue for the presence of antigens and migrate to lymphoid organs where antigenic peptides captured at the periphery can be presented to naive T lymphocytes (20). For the generation of CD4+ T lymphocytes, antigens are captured within the endocytic pathway, and during migration to the secondary lymphoid organs the dendritic cells mature and increase the expression of MHC class II–peptide complexes at the cell surface in order to efficiently induce T cell activation (22–24). How CD8+ T cells can be activated against antigens present in the periphery has been less well defined. For generation of CD8+ T lymphocytes, antigens captured in the periphery by professional antigen-presenting cells (APCs) have to acquire access to the MHC class I pathway. It is now becoming clear that a pathway does exist both in dendritic

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cells and macrophages to deliver exogenously captured antigens to the MHC class I-processing compartments referred to as cross-presentation (21, 25–27). Cross-presentation is crucial for the establishment of immunity against virus-infected and tumor-transformed cells as well as for the induction of tolerance (21, 28). The precise mechanisms involved in transfer of exogenous antigens to the MHC class I-processing and -presentation machinery are still unclear (6, 18, 29, 30, 70).

One internalization route used by macrophages and dendritic cells in particular to sample exogenous antigens to be cross-presented is macropinocytosis (17, 31–34). Macropinocytosis refers to the uptake of non-particulate material through the formation of 0.5- to 2-μm diameter vesicles in an actin-dependent process (35). Macropinosome formation starts at the cell periphery by extension of a large planar membrane ruffle (lamellipodium) that folds back to form the macropinosome (36–39). In macrophages and dendritic cells, formation of macropinosomes is a constitutive activity, which can be further enhanced by treatment with growth factors and activators of protein kinase C such as phorbol esters. Also in other cell types, macropinocytosis can be induced, although the significance of such macropinocytic events for internalization processes in these cells is unclear (35, 40, 41). Both induced and constitutive macropinosome formations are dependent on the activity of phosphatidylinositol (PI)-3-kinase (38, 42) and the activity of Rho family member Rac 1 (43, 44). Whereas activation of GTPase Rac 1 and its subsequent signaling to downstream effectors such as WAVE 2, an activator of the Arp2/3 complex (45, 46) or p21-activated kinases (47) is required for the rearrangement of the actin cytoskeleton, activated PI-3-kinase seems to be necessary for the completion of macropinosome formation (38). Macropinosomes remain separate from conventional endosomes (48), although fluid-phase markers internalized via macropinocytosis may eventually reach lysosomes (49).

Antigens internalized into APC via macropinocytosis gain access to the MHC class I-processing pathway (28, 32, 50) but the mechanisms involved in the transfer of macropinocytosed antigens to the cytosol remain unknown. As a first step toward defining the molecular events involved in the transfer of exogenous antigens to the MHC class I-processing and -presentation pathways, we analyzed the delivery of a model antigen, ovalbumin, to the class I-presentation pathway. We found that the steroid plasma membrane component cholesterol is essential for entry of ovalbumin into the class I pathway. Furthermore, chemical modification of ovalbumin to increase its affinity for cholesterol dramatically enhanced the capacity of the APCs to stimulate T lymphocytes.

Methods

Cells, antibodies and reagents

J774A.1 cells (American Type Tissue Collection) were maintained in DMEM (GIBCO) supplemented with 2 mM glutamine (GIBCO) and 10% FCS (GIBCO). Primary cells were obtained from isolated bone marrow of C57/BL6 mice. For generation of macrophages, the bone marrow cells were grown for 7 days in DMEM containing 10% FCS, 5% horse serum (GIBCO), 2 mM glutamine, 1 mM sodium pyruvate (GIBCO), 0.5 mM β-mercaptoethonal (GIBCO) and 30% L929-conditioned media. To obtain immature dendritic cells from bone marrow-derived precursor cells, DMEM was supplemented with 1% glutamine, 10% FCS and 25 ng ml^{-1} granulocyte macrophage colony-stimulating factor (Genzyme, Diagnostic). After 3 days of culture, 1.5 ng ml^{-1} interleukin-4 (R &D Systems) was added. FITC–Dextran (2 000 000 MW) was purchased from Molecular Probes. Methyl-β-cyclodextrin, ovalbumin, mevalonic acid, filipin complex III, horse radish peroxidase (HRP) and palmitic-acid-N-hydroxysuccinimid-ester (NHS–palmitic acid) were obtained from Sigma and phorbol myristate acetate (PMA) and lovastatin from Calbiochem.

Antibodies against lysosomal-associated membrane glycoprotein-1 (LAMP-1) (ID4B) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA. As a secondary reagent, anti-rat IgG (H + L)-568 (Molecular Probes) was used. Clathrin was stained using mouse anti-clathrin (Transduction Laboratories) and anti-mouse IgG1-TXR (Southern Biotechnology Association Inc.) antibodies.

Preparation of the cholesterol–methyl-β-cyclodextrin inclusion complex

The synthesis of the cholesterol–methyl-β-cyclodextrin complex was performed as described before (51). In brief, 30 mg cholesterol was dissolved in 2-propanol and added in small aliquots to a stirred solution of 1 mg methyl-β-cyclodextrin (5% w/v) on a water bath (80°C). Stirring was continued until everything was dissolved. After the removal of solvents by freeze drying, the cholesterol–methyl-β-cyclodextrin complexes were stored at room temperature (RT).

Cholesterol depletion and replenishment

Cholesterol depletion was performed as described in (56). In brief, cells were grown in the presence of 4 μM lovastatin (10 mM stock solution in 10% ethanol) and 250 μM mevalonate (J774A.1 for 60 h, bone marrow-derived macrophages and dendritic cells for 108 h) after which the residual plasma membrane cholesterol was extracted with methyl-β-cyclodextrin (J774A.1: 10 mM methyl-β-cyclodextrin for 45 min; bone marrow-derived macrophages: 20 mM methyl-β-cyclodextrin for 75 min; bone marrow-derived dendritic cells: 15 mM methyl-β-cyclodextrin for 45 min). All subsequent experiments were done in serum-free media. For the replenishment of cholesterol, depleted cells were incubated for 1 h in DMEM containing 6 mg ml^{-1} cholesterol–β-methyl-cyclodextrin complex.

Immunofluorescence

Control and cholesterol-depleted cells grown on glass coverslips were treated for 30 min with 10^{-7} M PMA (in the case of macrophages) followed by 7- or 12-min incubation with FITC–Dextran (1 mg ml^{-1}) or 0.1 mg ml^{-1} FITC-labeled ovalbumin/palmitoylated ovalbumin. Subsequently, cells were washed and fixed with 3% PFA. For staining, cells were permeabilized with 0.1% saponin in PBS for 20 min, blocked with 2% BSA in PBS for a further 20 min and labeled with

primary and secondary antibodies diluted in PBS/0.1% saponin/2% BSA for 30–45 min at RT. Internalization of HRP (5 mg ml^{-1}) was performed for 1 h at 37°C followed by a chase of 4 h. Cells were washed extensively, fixed in glutaraldehyde (0.5% w/v) at RT and internalized HRP was visualized by incubation with 0.5 mg ml^{-1} diaminobenzidine (Sigma) and 0.05% (v/v) H_2O_2 in PBS for 10 min. For palmitoylated HRP, internalization was performed for 1 h with 1 mg ml^{-1} of control or palmitoylated protein. Afterward, the cells were washed, fixed and permeabilized with 0.1% saponin for 20 min and internalized HRP was visualized as described before.

Quantitation of horse radish peroxidase (HRP) internalization

Control and cholesterol-depleted bone marrow-derived macrophages were incubated with 2 mg ml^{-1} HRP for 1 h at 37 or 0°C, washed and subsequently lysed in 20 mM HEPES, 0.1 M NaCl, 5 mM $MgCl_2$ and 1% (v/v) Triton-X100. The enzymatic activity of HRP in the lysates was determined by mixing 10 μl of cell lysate with 200 μl reaction buffer containing 0.342 mM *o*-dianisidine, 0.003% H_2O_2 (v/v), 50 mM sodium phosphate pH 5.0 and 0.3% (v/v) Triton-X100. After 5 min incubation at RT, the absorbance was measured at 455 nm and the obtained values, which correlate with the amount of HRP in the lysate, were related to the total protein amount in the lysates.

Flow cytometry

Macrophages were incubated with 0.1 mg ml^{-1} FITC-labeled ovalbumin or palmitoylated ovalbumin for 20 min at 37°C, washed, fixed with 3% PFA for 30 min on ice and subsequently analyzed by flow cytometry. Internalization of ovalbumin was measured as increase in fluorescence and expressed as mean fluorescence intensity. Shown are the mean values from three independent experiments.

Video microscopy

Control or cholesterol-depleted cells were placed under Zeiss Axiovert TV100 equipped with a thermostated (37°C) and CO_2-equilibrated chamber (10% CO_2). Thirty-minute video sequences were recorded using a digital camera (Hamamatsu Photonics Japan) and OpenLab software (version 2). Sequences were converted to QuickTime format.

Cross-presentation

Bone marrow-derived macrophages were seeded in 96-well plates (20 000 cells per well). After PMA stimulation and cholesterol extraction, cells were incubated with ovalbumin/palmitoylated ovalbumin or SIINFEKL/palmitoylated SIINFEKL peptide for 3 h. The non-internalized and non-bound antigens were removed by extensive washing and lymphocytes (total or $CD8^+$ cells) isolated from OT-1 mice (60) were added to the bone marrow-derived macrophages (ratio 5:1). T cell proliferation was measured after 3 days by labeling the proliferating cells with [methyl-^{3}H]thymidine (Amersham Pharmacia Biotech). Lactacystin treatment was performed by incubation of the cells with 10 μM lactacystin 1 h before and during the 3-h antigen pulse. Fixation with 1% PFA (30 min, 37°C) was done prior to incubation with the antigen. The mean values of one representative experiment are shown in Figs 4 and 5.

Palmitoylation of ovalbumin and HRP

Palmitoylation was performed following the method of Huang et al. (52). In brief, 5–30 mg proteins were dissolved in 1 ml of 20 mM NaPP, 150 mM NaCl, 2% deoxycholate pH 8.5. A 20 molar excess of NHS–palmitic acid was dissolved in 100 μl dioxane. Then 300 μl dimethyl sulfoxide (DMSO) and 100 μl dioxane containing the NHS–palmitic acid were slowly added to the protein solution. After overnight incubation, the reaction was stopped by addition of 1/100 volume of 1 M lysine and free NHS–palmitic acid was removed by gel filtration (PD 10 column, Amersham Bioscience). To separate palmitoylated from non-palmitoylated protein, hydrophobic interaction chromatography (HIC) was performed. Therefore, proteins were loaded onto a phenyl superose column (HR 10/10, Pharmacia), high salt buffer (20% buffer B, buffer A: 1.7 M ammonium sulfate, 50 mM sodium phosphate pH 7, buffer B: 50 mM sodium phosphate) allowing only the binding of palmitoylated protein. To elute column-bound palmitoylated protein, the salt concentration was gradually reduced (100% buffer B in 20 min). Palmitoylated proteins were analyzed by mass spectrometry or HPLC.

FITC labeling of ovalbumin and palmitoylated ovalbumin

Ovalbumin or palmitoylated ovalbumin was dissolved in PBS and a 5 molar excess of Oregon Green–NHS in DMSO was added to the protein solution. The reaction was performed at RT for 2 h under rotation. The free Oregon Green–NHS was removed afterward by gel filtration (PD10, Amersham Bioscience).

Results

Contribution of cholesterol to macropinosome formation

To gain insight into the delivery process of exogenous antigens to the MHC class I-presentation pathway, different professional APCs were incubated with FITC-labeled Dextran, a marker for macropinosomes (32, 34, 53). Both J774 cells and bone marrow-derived macrophages as well as bone marrow-derived dendritic cells contained large numbers of macropinosomes (Fig. 1A and B). As macropinosomes provide a port of entry to deliver components into the cell, similar to endosomes, the relation of these organelles with other endocytic compartments was examined. To that end, macrophages that had internalized FITC–Dextran via macropinocytosis were analyzed for the presence of different markers of the endosomal/lysosomal pathway, including clathrin and LAMP-1, none of which co-localized with macropinosomes. However, when cells were incubated with the cholesterol-binding compound filipin (54–56), all macropinosomes strongly labeled with filipin (Fig. 1B). This suggests that cholesterol is an important component of macropinosomes in macrophages and dendritic cells.

To analyze the contribution of cholesterol to macropinosome formation, the plasma membrane of macrophages and dendritic cells was depleted for cholesterol by pharmacological treatment with lovastatin (56). Living cells were observed under the microscope and macropinosome formation

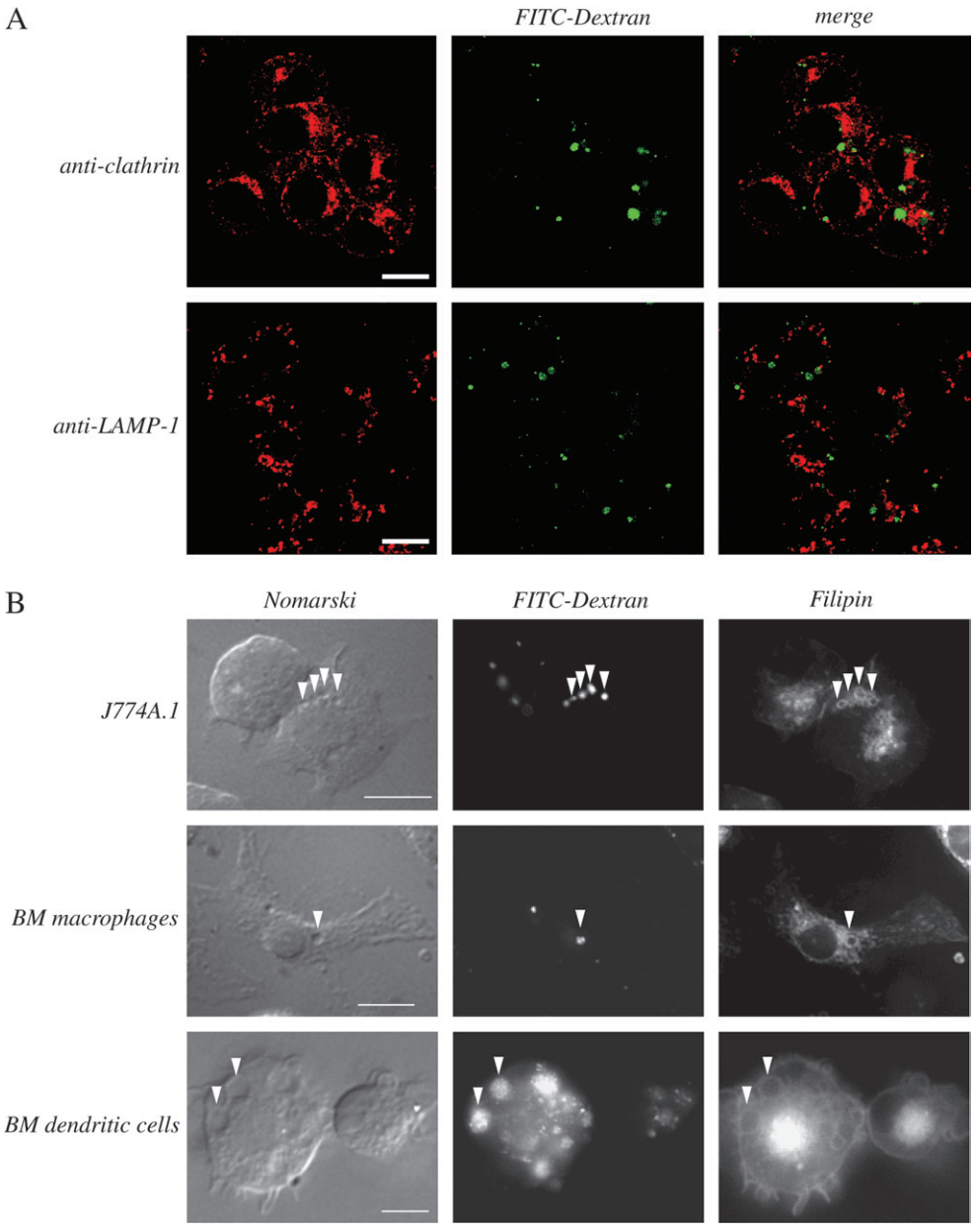
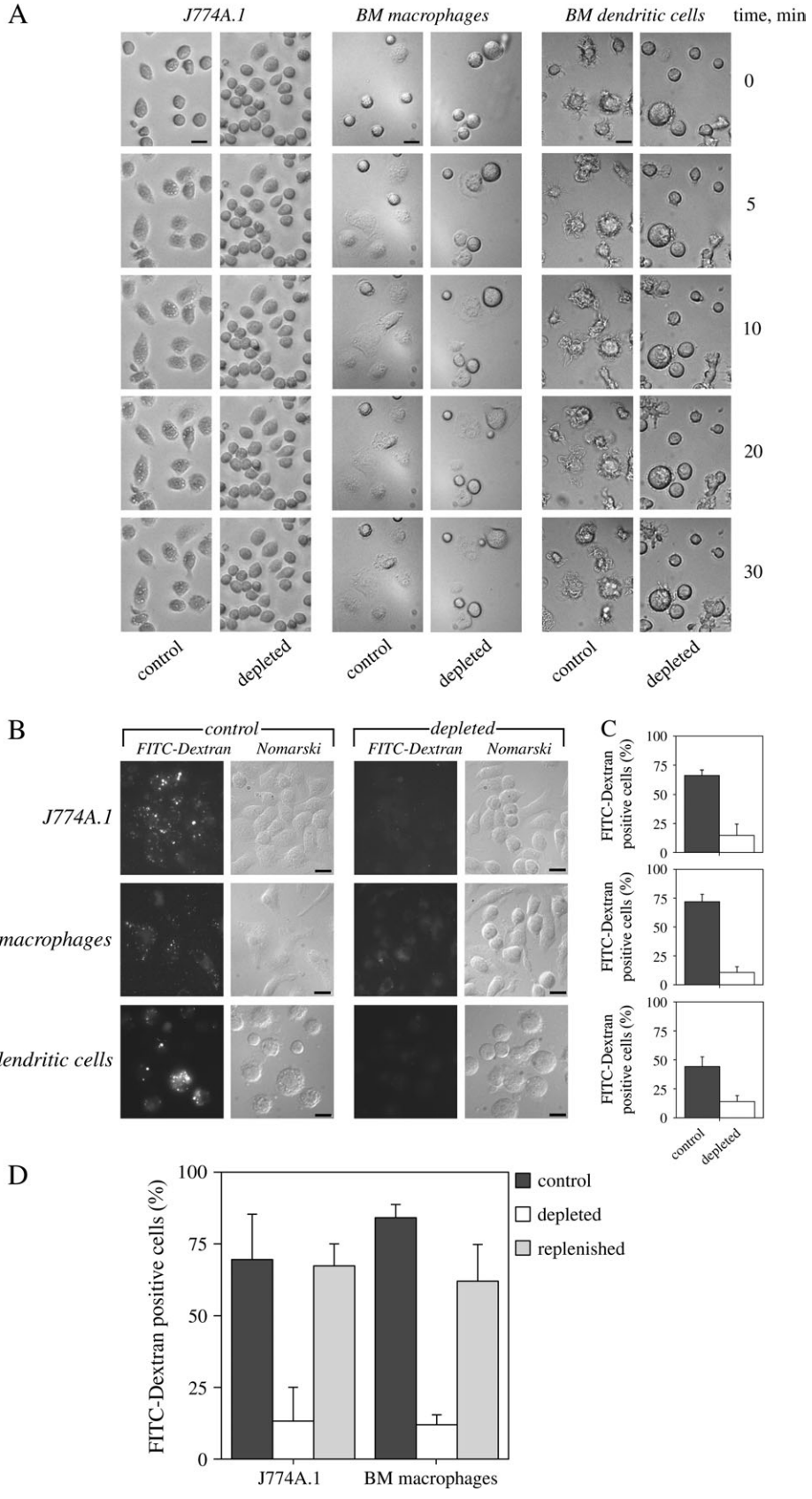


Fig. 1. Distribution of macropinocytosed FITC–Dextran in macrophages and dendritic cells. (A) Macrophages (J774A.1 cells) were incubated with FITC–Dextran for 7 min, fixed and stained for clathrin (upper panels) and LAMP-1 (lower panels), followed by Texas red-labeled secondary antibodies. (B) J774A.1 macrophages (upper panels), bone marrow (BM)-derived macrophages (middle panels) and BM-derived dendritic cells (lower panels) were incubated for 12 min with FITC–Dextran, fixed and labeled for cholesterol with filipin. Bar: 10 μm.

Fig. 2. Effect of cholesterol depletion on macropinocytosis. (A) J774A.1 cells (right panels), bone marrow (BM)-derived macrophages (middle panels) or bone marrow (BM)-derived dendritic cells (left panels) were left untreated (control) or cholesterol depleted (depleted) and placed under a 37°C-thermostated and CO_2-equilibrated microscope and subsequently 30-min video sequences were recorded. Shown are still images from the indicated times (see full video sequences at http://www2.biozentrum.unibas.ch/pieters/movies.html) Bar: 10 μm. (B and C) Control (left) or cholesterol-depleted (right) J774A.1 macrophages (upper panels), BM-derived macrophages (middle panels), or dendritic cells (lower panels) were incubated with FITC–Dextran for 12 min, fixed and analyzed by fluorescence microscopy. Bar: 10 μm. (C) For quantitation, cells ($n = 150$) were scored for the presence of internalized FITC–Dextran. Shown are mean values (±SD) from three experiments. (D) J774A.1 macrophages (left) or BM-derived macrophages (right) were left untreated (dark gray bars), depleted for cholesterol (light gray bars) or cholesterol-depleted and replenished by treatment for 1 h with cholesterol–methyl-β-cyclodextrin complex (white bars). Subsequently, cells were incubated for 12 min with FITC–Dextran. After fixation, the number of FITC–Dextran-positive cells was determined. Shown are mean values (±SD) from three experiments.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J774A.1
BM macrophages
BM dendritic cells
time, min
0
5
10
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FITC-Dextran
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FITC-Dextran
Nomarski
J774A.1
BM macrophages
BM dendritic cells
C
FITC-Dextran positive cells (%)
100
75
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control
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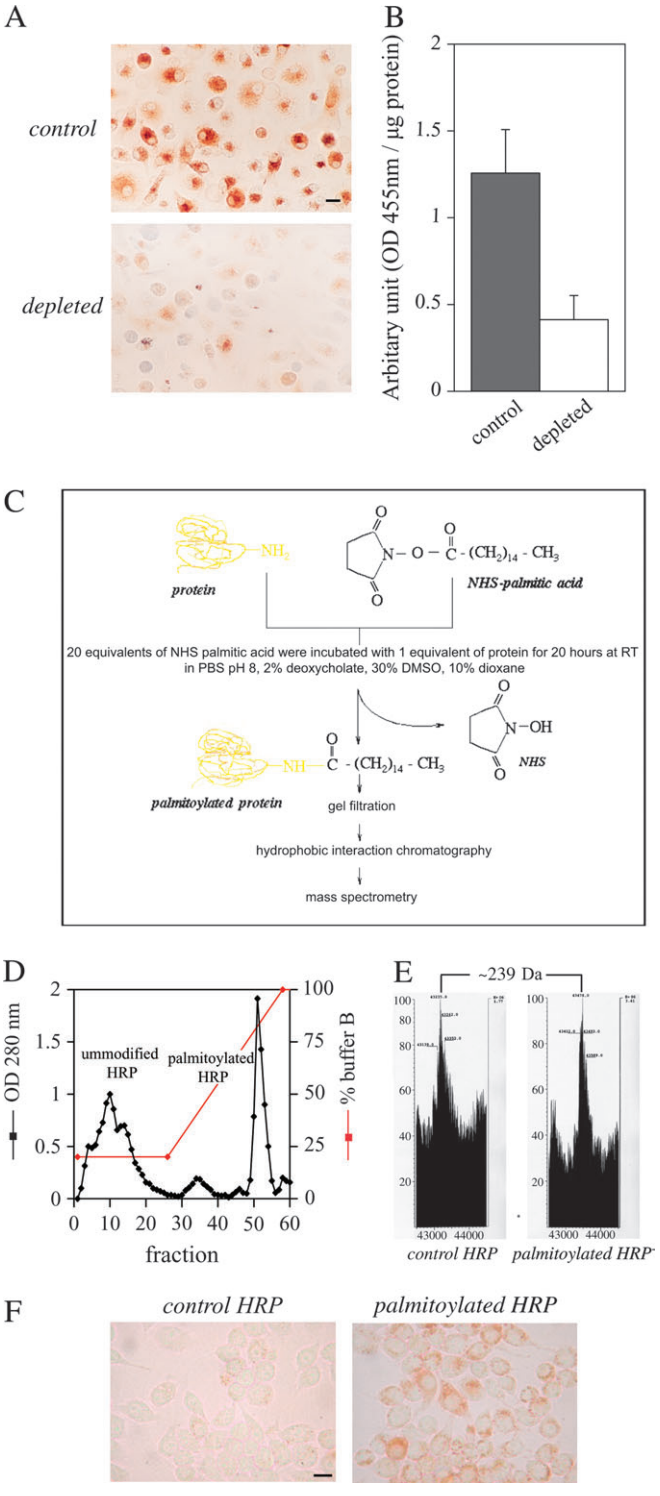
J774A.1
BM macrophages

was monitored using time-lapse video microscopy and 30-min video sequences of control and cholesterol-depleted cells were recorded. As shown in Fig. 2(A), whereas membrane ruffling occurred both in control as well as in cholesterol-depleted cells, macropinosome formation was abolished in cells depleted of cholesterol (see also movies at http://www2.biozentrum.unibas.ch/pieters/movies.html). To directly analyze internalization of the fluid-phase marker FITC–Dextran, control or cholesterol-depleted cells were incubated with FITC–Dextran for 12 min, fixed and observed under the fluorescence microscope. After cholesterol depletion, macrophages and dendritic cells were unable to internalize the fluid-phase marker, in contrast to control cells (Fig. 2B and C). Internalization of FITC–Dextran was fully restored when cholesterol-depleted cells were incubated with methyl-cyclodextrin–cholesterol complex (Fig. 2D), indicating that cholesterol is necessary for macropinosome formation.



Modulation of internalization by palmitoylation

The finding that macropinosomes are cholesterol-rich structures, lead us to explore the possibility that enhancement of the affinity of antigens for cholesterol improves internalization. One modification that targets proteins to cholesterol-enriched sites in the plasma membrane is palmitoylation (57–59). As a model protein, the behavior of HRP after modification by palmitoylation was investigated. HRP was internalized into macrophages via macropinocytosis and internalization was inhibited following cholesterol depletion (Fig. 3A and B).

To modify HRP by palmitoylation, HRP was incubated for 20 h with NHS–palmitic acid (Fig. 3C). After palmitoylation, the product was purified by HIC (Fig. 3D). Mass spectrometry analysis showed a mass shift of 239 Da upon palmitoylation consistent with the addition of one palmitic acid residue. (Fig. 3E). Internalization of palmitoylated HRP in macrophages can be blocked by cholesterol depletion, indicating that palmitoylated HRP is taken up via macropinocytosis (data not shown). Furthermore, internalization of palmitoylated HRP is far more efficient compared with non-modified HRP, which was barely detectable at the same concentration (Fig. 3F). Together, these results suggest that palmitoylation leads to an enhanced uptake of exogenous proteins via macropinocytosis.

Role for cholesterol in cross-presentation

Given the role of cholesterol in macropinocytosis, the involvement of cholesterol in cross-presentation was investigated. For

Fig. 3. Palmitoylation of HRP. (A) Control (upper panel) or cholesterol-depleted (lower panel) bone marrow (BM)-derived macrophages were incubated for 1 h with HRP (5 mg ml^{-1}) and chased for 4 h followed by fixation. The internalized HRP was visualized as described in Methods. Bar: 10 µm. (B) Control (dark gray bar) or cholesterol-depleted (white bar) BM-derived macrophages were incubated for 1 h with HRP (2 mg ml^{-1}). After washing, cells were lysed and the amount of internalized HRP in the lysate was determined as described in Methods. Shown are the mean values (±SD) from three experiments. (C) Palmitoylation procedure of HRP. HRP was incubated with an NHS-activated form of palmitic acid for 24 h allowing the formation of amid bounds between the acid and primary amino groups of the protein. The palmitoylated protein was desalted and purified with HIC. (D) Separation of palmitoylated from unmodified HRP by HIC. The reaction product as described in panel C was loaded onto phenyl superose and palmitoylated HRP was eluted after decreasing the salt concentration by increasing the percentage of buffer B (see Methods). (E) Mass spectrometry analysis (MALDI-TOF) of control HRP (left panel) and palmitoylated HRP (right panel). The mass shift of 239 Da corresponds to the attachment of one palmitic acid chain to the protein. (F) J774A.1 macrophages were incubated for 1 h with HRP (1 mg ml^{-1}, left panel) or palmitoylated HRP (1 mg ml^{-1}, right panel) fixed and permeabilized. Internalized HRP was visualized as in panel A. Bar: 10 µm.

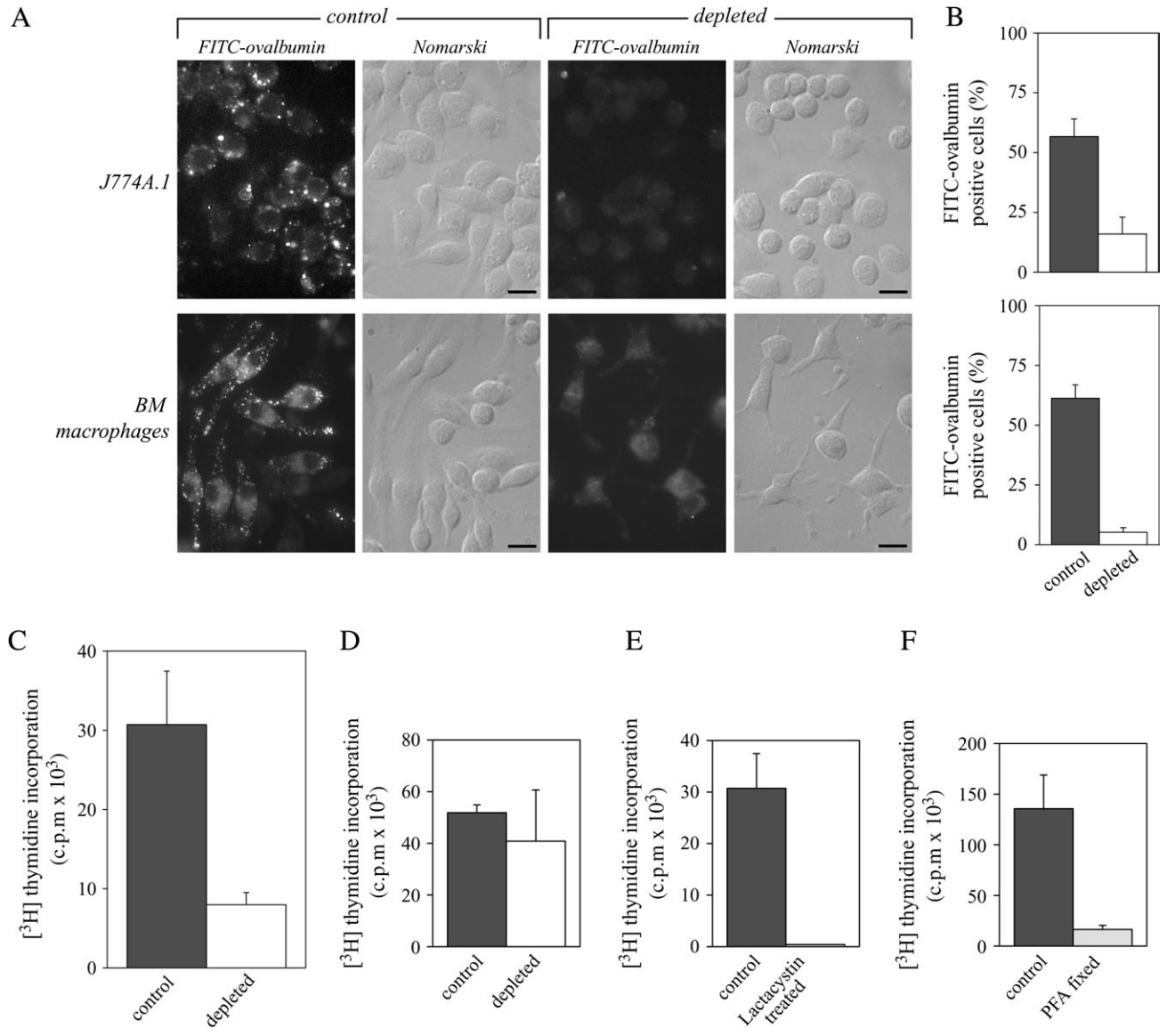


Fig. 4. Uptake and cross-presentation of ovalbumin in control and cholesterol-depleted cells. (A and B) Macrophages (top panels: J774 A.1; bottom panels: bone marrow-derived macrophages) were left untreated (control) or depleted for cholesterol (depleted) and incubated with FITC–ovalbumin (0.1 mg ml^{-1}) for 12 min, fixed and analyzed by fluorescence microscopy. Bar: 10 μm. (B) Quantitation was carried out after fixation. Values represent the percentages of cells having internalized FITC–ovalbumin. Shown are the mean values (±SD, $n = 50$) from three experiments. (C–F). Bone marrow-derived macrophages were left untreated (dark gray bars) or depleted (white bars) for cholesterol and incubated with ovalbumin (10 mg ml^{-1}) (C, E and F) or SIINFEKL peptide (10 mM) (D) for 3 h. In (E), macrophages were treated with lactacystin. In (F), macrophages were fixed with PFA prior to addition of ovalbumin (5 mg ml^{-1}). Cells were washed and T cells specific for the SIINFEKL epitope isolated from OT-1 mice were added. T cell proliferation was measured after 3 days as described in Methods.

that purpose, we made use of the model antigen ovalbumin. Ovalbumin labeled with FITC, when added to macrophages, was efficiently internalized into macropinosomes. Conversely, internalization of FITC–ovalbumin in cholesterol-depleted cells was greatly reduced (Fig. 4A and B).

To analyze cross-presentation, control or cholesterol-depleted macrophages were incubated with ovalbumin for 3 h and washed, and the cell surface display of the ovalbumin-derived MHC class I SIINFEKL epitope was measured using T cells obtained from OT-1 mice that express a TCR specific for the ovalbumin-derived SIINFEKL peptide (60). The capacity of the ovalbumin-loaded macrophages to stimulate OT-1 T cells was quantified by measuring T cell proliferation, which is a direct result of T cell activation initiated by the recognition of the appropriated antigen on the surface of the APC.

Whereas control macrophages stimulated T cell proliferation, depletion of macrophages for cholesterol resulted in a severely reduced T cell activation (Fig. 4C). To analyze whether cholesterol depletion compromises the ability to present the SIINFEKL peptide *per se*, untreated and cholesterol-depleted macrophages were pulsed with SIINFEKL peptide that does not require internalization prior to presentation to

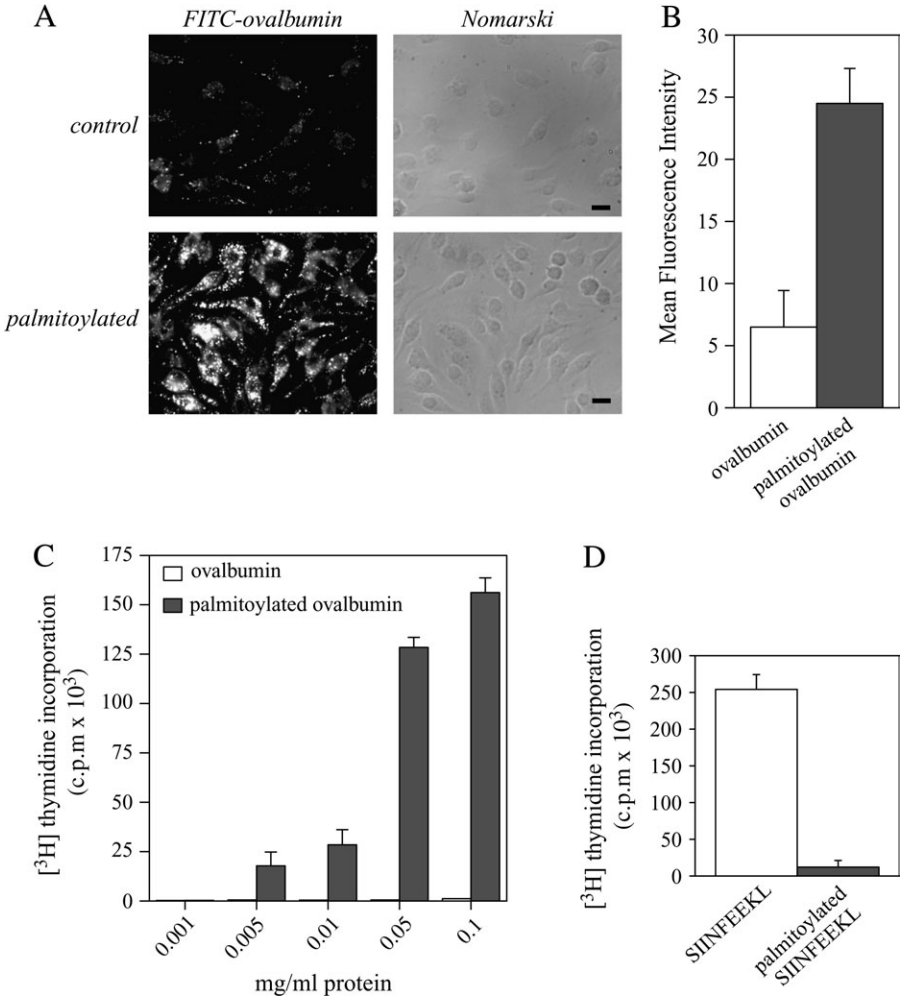


Fig. 5. Effect of palmitoylation on internalization and cross-presentation. (A) Bone marrow-derived macrophages were incubated with 0.1 mg ml^{-1} ovalbumin (upper panel) or palmitoylated ovalbumin (lower panel) that was labeled with FITC for 12 min, followed by fixation and analysis by fluorescence microscopy. Bar: 10 μm. (B) Bone marrow-derived macrophages were incubated with FITC-labeled ovalbumin (white bars) or palmitoylated ovalbumin (dark gray bars) for 20 min at 37°C, fixed and protein internalization analyzed by FACS. Shown are mean values (±SD) from three experiments. (C) Bone marrow-derived macrophages were incubated for 3 h with ovalbumin (white bars) or palmitoylated ovalbumin (dark gray bars). Cells were washed and T cells specific for the SIINFEKL epitope isolated from OT-1 mice were added, and T cell proliferation was measured as described in Methods. (D) Bone marrow-derived macrophages were incubated with 10 mM of SIINFEKL peptide or palmitoylated SIINFEKL peptide for 3 h.

T lymphocytes. As shown in Fig. 4(D), both in control or cholesterol-depleted macrophages, MHC class I presentation of exogenously added SIINFEKL peptide was comparable. Ovalbumin was indeed presented after cross-presentation, as the inclusion of the proteasome inhibitor lactacystin or fixation of the macrophages with PFA abolished T cell proliferation (Fig. 4E and F).

Modulation of cross-presentation by palmitoylation of ovalbumin

Given the dependence of cross-presentation on cholesterol, we analyzed whether modification of the antigen by palmitoylation could modulate cross-presentation. To that end, ovalbumin was palmitoylated as described and the capacity of macrophages to internalize and cross-present palmitoylated ovalbumin was examined. As shown in Fig. 5(A), internalization of FITC-labeled palmitoylated ovalbumin was increased compared with non-modified FITC–ovalbumin. Quantitation by flow cytometry suggested an at least 4-fold increase. However, when macrophages were pulsed with different concentrations of ovalbumin or palmitoylated ovalbumin, T cell proliferation was greatly enhanced following internalization of palmitoylated ovalbumin as compared with T cell proliferation induced after internalization of the same concentrations of ovalbumin (Fig. 5C). The enhanced T cell proliferation was due to internalization and processing of the palmitoylated ovalbumin, as palmitoylated SIINFEKL peptide was far less potent in the stimulation of T cell proliferation (Fig. 5D) as reported before (61). Together, these results show that modification of antigens via palmitoylation increased the ability to be internalized via macropinocytosis, processed intracellularly and presented on MHC class I molecules to T lymphocytes.

Discussion

Initiation of the immune response against infectious organisms occurs through the presentation of antigenic peptides by professional APCs, such as macrophages and dendritic cells. Elimination of viruses occurs largely through the activity of CTLs that become activated after presentation of viral antigens on MHC class I molecules. In recent years, it is becoming clear that these MHC class I-restricted antigens not only derive from endogenously synthesized proteins but that also exogenous proteins are a source for antigenic peptides presented via MHC class I (28, 30, 62). This 'cross-presentation' of exogenous antigens ensures the generation of an immune response when APCs are not infected by viruses itself and is thought to be important in the establishment of an immune response against tumors. In professional APCs such as macrophages and dendritic cells, one mechanism to internalize antigens for cross-presentation occurs via macropinocytosis (63). In this work, we show that internalization of antigens into macrophages and dendritic cells via macropinocytosis required the presence of cholesterol. Furthermore, modification of the model antigen ovalbumin with palmitoylation dramatically increased cross-presentation. These results suggest that modification of antigens to increase their affinity for cholesterol may be exploited to enhance the activation of CD8+ lymphocytes.

What role does cholesterol play in macropinosome formation? Phagocytosis is not affected in cholesterol-depleted macrophages (56) and therefore cholesterol is not necessary for the membrane curvature during the process of macropinosome formation. Recent findings in the epidermoid carcinoma cell line A431 showed the dependence of cholesterol for the plasma membrane localization of Rac 1 (41). Cholesterol depletion of A431 cells prevented plasma membrane localization of Rac 1 upon phorbol ester treatment thereby blocking membrane ruffling and macropinosome formation. In macrophages and dendritic cells, depletion of cholesterol abolishes macropinosome formation but does not affect membrane ruffling which is known to be independent of Rac 1 (44). Therefore, cholesterol depletion may inhibit macropinosome formation by blocking plasma membrane localization of Rac 1 also in professional APCs such as macrophages and dendritic cells.

Besides a role for cholesterol in the internalization process of soluble antigens into macropinosomes, it is likely that there is an additional role for cholesterol in the cytosolic translocation of these antigens to allow cross-presentation. This is based on our finding that while internalization of palmitoylated antigen was increased ~4-fold as determined by flow cytometry, presentation of ovalbumin-derived peptide was increased up to ~200-fold when the palmitoylated form of ovalbumin was administered. Cross-presentation of ovalbumin is dependent on the functioning of the proteasome, suggesting that translocation to the cytosol is required, but how exactly antigens are entering the cytoplasm for processing and presentation on MHC class I molecules remains unclear.

Several models to explain MHC class I presentation of exogenous antigens have been put forward. First, antigens could be internalized through different forms of endocytosis, including phagocytosis and macropinocytosis, followed by antigen degradation within such organelles and antigenic peptide loading on recycling MHC class I molecules. Second, antigens could be transferred to the cytosol where proteolytic activity of the proteasome would generate antigenic peptides to be translocated into the ER followed by presentation on ER-resident MHC class I molecules (18, 64).

Cross-presentation is blocked when APCs are incubated in the presence of proteasome inhibitors (32, 65). As proteasomes are not known to reside within endocytic organelles, this suggests that cytosolic translocation of the antigen has to occur in order to generate the appropriate peptides. How do protein antigens acquire access from endosomal organelles such as phagosomes and macropinosomes to the cytosol? One recently proposed possibility involves the same machinery that is responsible for translocation of misfolded proteins from the ER, namely the Sec61 translocon. Interestingly, in *Dictyostelium discoideum*, several resident ER proteins are necessary for phagocytosis (66). More recently, based on the localization of resident ER proteins within phagosomes (67), it has been proposed that phagosomes intersect with the ER. The ER elements could thereby provide most of the processing and presentation components required for MHC class I-restricted antigen presentation (29, 62, 68, 69). However, the concept of ER-mediated phagocytosis is questioned by a recent report where a contribution of the ER to the phagosome formation was not observed (70).

Whether or not molecules of the Sec61 translocation complex are involved in transport of antigenic peptides generated by the proteasome as well as import of antigens into the cytosol is unknown. Interestingly, cholesterol renders the Sec61 translocon machinery unable to recognize and/or initiate translocation of nascent polypeptide chains (71). As import of antigens from macropinosomes to the cytosol is topologically similar to retro translocation, cholesterol may play a role in ensuring cytosolic import of substrates, while blocking the activity of the Sec61 complex for passage of nascent chains.

The requirement for cholesterol in delivering exogenous antigens to the MHC class I-processing and -presentation pathways could be utilized to enhance the immune response against defined antigens. As shown here, palmitoylation of ovalbumin to increase its affinity for cholesterol (58, 59) dramatically enhanced the capacity of APCs loaded with this modified antigen to stimulate T lymphocytes. This is in contrast to the capacity of palmitoylated peptides that do require internalization and processing to trigger T cell activation, which is similar to or even lower than the non-modified peptides (61). The increase in T cell stimulatory capacity far exceeded the enhanced internalization of palmitoylated ovalbumin, suggesting that cholesterol plays an important role in the actual translocation process.

The potential of palmitoylation to enhance the immunity against exogenous antigens could be used for vaccine development against viruses and tumors (72). This approach might especially be useful when using protein antigens which, compared with peptides, have a greatly increased stability *in vivo*.

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Abbreviations

APC	antigen-presenting cell
DMSO	dimethyl sulfoxide
ER	endoplasmic reticulum
HIC	hydrophobic interaction chromatography
LAMP-1	lysosomal-associated membrane glycoprotein-1
PI	phosphatidylinositol
PMA	phorbol myristate acetate
RT	room temperature

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