articles

molecular pharmaceutics

Differential Cytosolic Delivery and Presentation of Antigen by Listeriolysin O-Liposomes to Macrophages and Dendritic Cells

Ethan M. Stier,[†] Manas Mandal,[‡] and Kyung-Dall Lee*

Department of Pharmaceutical Sciences, University of Michigan, 428 Church Street, Ann Arbor, Michigan 48109-1065

Received November 15, 2004

Abstract: Delivery of antigenic protein to the cytosol of antigen-presenting cells (APCs), such as macrophages (MΦ) and dendritic cells (DCs), is required for an efficient CD8 T-cell-mediated immune response. We have previously shown that co-encapsulation of antigenic protein inside pH-sensitive liposomes with listeriolysin O (LLO), a pore-forming protein of *Listeria monocytogenes*, generates efficient major histocompatibility complex class I (MHC I)-restricted immune responses both *in vitro* and *in vivo*. In this study, we sought to analyze the relative efficiency of LLO-mediated cytosolic delivery of liposomal antigen in two important APCs, macrophages and dendritic cells, by examining the sequential steps involved in antigen presentation to T-cells in cultured mouse bone marrow-derived MΦs (BMMΦs) and DCs (BMDCs). BMMΦs overall presented liposomal antigen better than BMDCs at a given concentration of liposomal antigen incubated with cells, and the trend was also observed after the presentation was normalized by the uptake of antigen. When soluble antigen was directly introduced into the cytosol, however, BMDCs presented the antigen more efficiently than BMMΦs. In addition, when the APCs were externally loaded with the antigenic peptide of the protein, BMDCs displayed a higher level of cell surface MHC I−peptide complexes and presented the peptide more efficiently than BMMΦs. These results combined together suggest that LLO-mediated release of liposomal antigen from the endosomal/lysosomal compartment may be more pronounced in BMMΦs than in BMDCs, and further implicates differential activity of LLO and varying efficiency of LLO-mediated endosomal escape in different antigen-presenting cell types.

Keywords: Antigen delivery; MHC I; dendritic cells; macrophages; liposomes; listeriolysin O

Introduction

A strong antigen-specific cytotoxic T lymphocyte (CTL) response is considered to be critical in the prevention and/or treatment of a number of disease states, including viral infection, cancer, and certain types of bacterial infection.[1−4]

* To whom correspondence should be addressed: Department of Pharmaceutical Sciences, University of Michigan, 428 Church St., Ann Arbor, MI 48109-1065. Telephone: (734) 647-4941. Fax: (734) 615-6162. E-mail: kdlee@umich.edu.

[†] Present address: Office of Generic Drugs, Center for Drug Evaluation and Research, Food and Drug Administration, 7520 Standish Pl., Rockville, MD 20855.

[‡] Present address: Center for Molecular Medicine and Infectious Disease, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, 1410 Prices Fork Rd., Blacksburg, VA 24061.

(1) Oldstone, M. The role of cytotoxic T lymphocytes in infectious disease: History, criteria, and state of the art. *Curr. Top. Microbiol. Immunol.* **1994**, *189*, 1−8.

(2) Rock, K. L.; Goldberg, A. L. Degradation of cell proteins and the generation of MHC class I-presented peptides. *Annu. Rev. Immunol.* **1999**, *17*, 739−779.

(3) San Mateo, L. R.; Chua, M. M.; Weiss, S. R.; Shen, H. Perforin-mediated CTL cytolysis counteracts direct cell−cell spread of *Listeria monocytogenes*. *J. Immunol.* **2002**, *169*, 5202−5208.

(4) Shimizu, K.; Thomas, E. K.; Giedlin, M.; Mule, J. J. Enhancement of tumor lysate- and peptide-pulsed dendritic cell-based vaccines by the addition of foreign helper protein. *Cancer Res.* **2001**, *61*, 2618−2624.

10.1021/mp049896v CCC: $30.25
Published on Web 12/16/2004

In priming CTLs, antigen-presenting cells (APCs) play a crucial role by processing and presenting epitopes derived from cytosolic antigen to CD8+ T-cells in a major histocompatibility complex I (MHC I)-dependent manner.[2,5]

Typically, exogenously administered proteins do not efficiently enter the cytosol because of poor permeability across the plasma membrane and endosomal/lysosomal membrane.[6] Instead, after uptake by cells through endocytosis, they are typically degraded in the endosomal/lysosomal compartment and thus presented predominantly in a MHC II-restricted manner. In contrast, antigenic protein within the cytosol is processed into peptide fragments, which are subsequently transported into the endoplasmic reticulum (ER) where they bind to MHC I molecules, and are then displayed on the cell surface as peptide−MHC I complexes.[7] Thus, induction of an efficient MHC I-dependent immune response requires augmented delivery of antigenic protein to the cytosol of APCs.[8−11]

We have previously reported an approach designed for such enhanced cytosolic delivery of exogenous protein by combining liposomes with a strategy that is based on the endosome-perforating (endosomolytic) mechanism used by *Listeria monocytogenes* (LM) to invade the cytosol of a host cell.[12,13] After binding and internalization via the endosomal/lysosomal pathway, LM secretes LLO, a 58 kDa protein that forms pores within the endosomal/lysosomal membrane, enabling escape of the bacterium into the cytosol.[14] LLO has been shown to have optimal activity at the acidified pH of the endosomal/lysosomal compartment.[15,16] It was demonstrated that pH-sensitive liposomes containing purified LLO (LLO-liposomes) and ovalbumin (OVA) efficiently induced MHC I-restricted OVA-specific antigen presentation *in vitro* in an LLO-dependent manner, and a robust OVA-specific CTL response *in vivo* that was clearly augmented by LLO.[12,13]

Although a strong antigen-specific CTL response was observed using LLO-liposome-mediated antigen delivery *in vivo*, little is known about the relative contribution from different types of APCs to the presentation of antigen encapsulated in LLO-liposomes. We sought to address this issue by first investigating *in vitro* the relative efficiency of antigen delivery and presentation by two major APCs, DCs and MΦs. DCs are reported to be highly efficient at capturing antigen, and are the only APC reported to be capable of activating a naive T-cell.[17,18] MΦs can efficiently present antigen to T-cells, are highly efficient at capturing liposomal antigen, and may also cross-present antigen to DCs *in vivo*.[19,20] Some studies have compared DCs and MΦs using assays that require high costimulatory activity to the T-cells, while we assessed antigen presentation in this study using a well-established *in vitro* assay that generates readout signals independent of the costimulatory activity.[21,22]

LLO-liposome-mediated delivery of antigen to the cytosol, leading to MHC I-dependent presentation, involves a sequence of complex steps. In the first step, the liposomal formulation binds to the cell surface and enters the cell via endocytosis. In the second step, LLO and antigen are released from the destabilized liposomes in the endosomal/lysosomal

(5) Germain, R. N. MHC-dependent antigen processing and peptide presentation, providing ligands for T lymphocyte activation. *Cell* **1994**, *76*, 287−299.
(6) Provoda, C. J.; Lee, K. D. Bacterial pore-forming hemolysins and their use in the cytosolic delivery of macromolecules. *Adv. Drug Delivery Rev.* **2000**, *41*, 209−221.
(7) York, I. A.; Goldberg, A. L.; Mo, X. Y.; Rock, K. L. Proteolysis and class I major histocompatibility complex antigen presentation. *Immunol. Rev.* **1999**, *172*, 49−66.
(8) Moore, M. W.; Carbone, F. R.; Bevan, M. J. Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell* **1988**, *54*, 777−785.
(9) Harding, C. V.; Collins, D. S.; Kanagawa, O.; Unanue, E. R. Liposome-encapsulated antigens engender lysosomal processing for class II MHC presentation and cytosolic processing for class I presentation. *J. Immunol.* **1991**, *147*, 2860−2863.
(10) Nair, S.; Zhou, F.; Reddy, R.; Huang, L.; Rouse, B. T. Soluble proteins delivered to dendritic cells via pH-sensitive liposomes induce primary cytotoxic T lymphocyte responses in vitro. *J. Exp. Med.* **1992**, *175*, 609−612.
(11) Kovacsovics-Bankowski, M.; Clark, K.; Benacerraf, B.; Rock, K. L. Efficient major histocompatibility complex class I presentation of exogenous antigen upon phagocytosis by macrophages. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 4942−4946.
(12) Lee, K. D.; Oh, Y. K.; Portnoy, D. A.; Swanson, J. A. Delivery of macromolecules into cytosol using liposomes containing hemolysin from *Listeria monocytogenes*. *J. Biol. Chem.* **1996**, *271*, 7249−7252.
(13) Mandal, M.; Lee, K. D. Listeriolysin O-liposome-mediated cytosolic delivery of macromolecule antigen in vivo, enhancement of antigen-specific cytotoxic T lymphocyte frequency, activity, and tumor protection. *Biochim. Biophys. Acta* **2002**, *1563*, 7−17.
(14) Portnoy, D. A.; Auerbuch, V.; Glomski, I. J. The cell biology of *Listeria monocytogenes* infection, the intersection of bacterial pathogenesis and cell-mediated immunity. *J. Cell Biol.* **2002**, *158*, 409−414.
(15) Portnoy, D. A.; Jone, S. The cell biology of *Listeria monocytogenes* infection (escape from a vacuole). *Ann. N.Y. Acad. Sci.* **1994**, *730*, 15−25.
(16) Beauregard, K. E.; Lee, K. D.; Collier, R. J.; Swanson, J. A. pH-dependent perforation of macrophage phagosomes by listeriolysin O from *Listeria monocytogenes*. *J. Exp. Med.* **1997**, *186*, 1159−1163.
(17) Banchereau, J.; Briere, F.; Caux, C.; Davoust, J.; Lebecque, S.; Liu, Y. J.; Pulendran, B.; Palucka, K. Immunobiology of dendritic cells. *Annu. Rev. Immunol.* **2000**, *18*, 767−811.
(18) Mellman, I.; Turley, S. J.; Steinman, R. M. Antigen processing for amateurs and professionals. *Trends Cell Biol.* **1998**, *8*, 231−237.
(19) Papahadjopoulos, D.; Allen, T. M.; Gabizon, A.; Mayhew, E.; Matthay, K.; Huang, S. K.; Lee, K. D.; Woodle, M. C.; Lasic, D. D.; Redemann, C.; et al. Sterically stabilized liposomes, improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 11460−11464.
(20) Harshyne, L. A.; Watkins, S. C.; Gambotto, A.; Barratt-Boyes, S. M. Dendritic cells acquire antigens from live cells for cross-presentation to CTL. *J. Immunol.* **2001**, *166*, 3717−3723.
(21) Harding, C. V. Techniques for studying phagocytic processing of bacteria for class I or II MHC-restricted antigen recognition by T lymphocytes. *Methods Cell Biol.* **1994**, *45*, 313−326.
(22) Rock, K. L.; Rothstein, L.; Gamble, S. Generation of class I MHC-restricted T-T hybridomas. *J. Immunol.* **1990**, *145*, 804−811.

acidic pH compartment and the antigen is released into the cytosol of the APC upon formation of the pore by LLO. In the third step, the antigen released into the cytosol is processed into peptide fragments, transported into the endoplasmic reticulum (ER), and loaded onto MHC I molecules. Finally, in the fourth step, the antigen-derived peptides complexed with MHC I molecules are presented on the surface of the APC to be recognized by cognate T-cells.

In this study, we assessed the efficiency and differences, between BMMΦs and BMDCs, in the uptake of the liposomal formulation (step 1), processing of antigenic protein in the cytosol (step 3), and presentation of peptide antigen to T-cells (step 4). As we investigated the overall extent of antigen presentation, along with our investigation of steps 1, 3, and 4, we were able to assess the relative rate of step 2, endosomal escape into cytosol, in MΦs and DCs. Our results demonstrate the relative efficiency for each step is dependent on the cell types. BMMΦs, as compared with BMDCs, are more efficient in acquiring liposomal antigen as well as in overall presentation of liposomal antigen to T-cells per antigen uptake. However, BMDCs are more efficient in presenting cytosolic antigenic protein directly introduced by trituration as well as antigenic peptide loaded externally. These results suggest that that LLO-mediated delivery of protein into cytosol, defined as delivery of the antigen from the endosome per unit antigen uptake, is more efficient in BMMΦs than in BMDCs. The data also indicate that LLO may have preferential activity in the endosomal/lysosomal compartment of BMMΦs as compared to BMDCs.

Materials and Methods

Animals, Cell Lines, Media, and Reagents. C57BL/6J mice (female, 4−6 weeks old; Jackson Laboratories) used in this study were handled according to the Institutional Guidelines. OVA peptide SIINFEKL-specific $CD8^+$ T-cell hybridoma [CD8 OVA T1.3, H-$2K^b$-restricted, from C. Harding (Case Western Reserve University, Cleveland, OH)] was maintained in complete Dulbecco's minimal essential medium (DMEM).[21] Hybridomas producing monoclonal antibodies (mAbs) NLDC-145, specific for DEC-205, and 25-D1.16, specific for the H-$2K^b$−SIINFEKL complex, were gifts from R. M. Steinman (Rockefeller University, New York, NY) and R. N. Germain (National Institutes of Health, Bethesda, MD), respectively.[23,24] Both cell lines were grown in RPMI-1640 with 10% fetal bovine serum (FBS).

Cell culture media were obtained from Gibco (Grand Island, NY) unless specified. Dendritic cell media (DCM) consisted of 10% FBS, 1 mM sodium pyruvate, 2 mM glutamine, 100 μg/mL streptomycin, 100 units/mL penicillin, and 5×10^{-5} M β-2-mercaptoethanol (Sigma, St. Louis, MO), 10 ng/mL GM-CSF (Peprotech), and 10 ng/mL IL-4 (Peprotech) in RPMI-1640. ACK lysis buffer consisted of 0.15 M ammonium chloride, 10 mM potassium bicarbonate, and 0.1 mM EDTA (Sigma) at pH 7.2. PDE buffer consisted of Ca^{2+} and Mg^{2+}-free PBS containing 3 mM EDTA (Sigma).

OVA peptide SIINFEKL (amino acids 257−264) was synthesized by Research Genetics, dissolved in PBS, and stored at −80 °C in aliquots until it was used. All other chemicals were obtained from Fisher Scientific unless otherwise noted.

Purification of LLO. Recombinant LLO was purified from *Escherichia coli* strain BL21(DE3) transformed with the pET29b vector expressing LLO with a C-terminal six-histidine tag as previously described.[13] The protein yield was measured using the BCA assay (Pierce, Rockford, IL), and protein purity was analyzed using SDS−PAGE. Hemolytic activity was measured using the sheep red blood cell-based hemolysis assay as previously described.[13]

Preparation of OVA/LLO and Horseradish Peroxidase (HRP) Liposomes. OVA/LLO or HRP liposomes were prepared with phosphatidylethanolamine (PE) (Avanti, Alabaster, AL) and cholesterylhemisuccinate (CHEMS) (Sigma) in a 2:1 molar ratio using the thin film method as previously described.[13] Briefly, OVA (Sigma) and LLO were encapsulated inside liposomes at 20 and 0.25 mg/mL, respectively, and HRP (Sigma) was encapsulated in liposomes at 3 mg/mL. Unencapsulated protein was removed by purification on a Sepharose CL-4B column (Amersham Pharmacia, Uppsala, Sweden). The amount of encapsulated protein was determined by SDS−PAGE and densitometry. The size of liposomes was measured by quasi-elastic light scattering using a particle sizer (Zetasizer, Nicomp). The concentration of phosphate was determined using the method of Bartlett.[25]

Generation of BMDCs. A well-established method for culturing BMDCs was used with minor modifications.[26,27] Bone marrow cells (BMCs) were collected from the long bones of mice. The cells were pipetted to form a single cell suspension, and cell debris was removed by gentle centrifugation. Red blood cells were removed by treating BMCs with ACK lysis buffer, and cells were washed three times with RPMI-1640 complete. Then BMCs were resuspended in

(23) Inaba, K.; Swiggard, W. J.; Inaba, M.; Meltzer, J.; Mirza, A.; Sasagawa, T.; Nussenzweig, M. C.; Steinman, R. M. Tissue distribution of the DEC-205 protein that is detected by the monoclonal antibody NLDC-145. I. Expression on dendritic cells and other subsets of mouse leukocytes. *Cell. Immunol*. **1995**, *163*, 148−156.

(24) Porgador, A.; Yewdell, J. W.; Deng, Y.; Bennink, J. R.; Germain, R. N. Localization, quantitation, and in situ detection of specific peptide-MHC class I complexes using a monoclonal antibody. *Immunity* **1997**, *6*, 715−726.

(25) Bartlett, G. R. *J. Biol. Chem.* **1959**, *234*, 466−468.

(26) Inaba, K.; Inaba, M.; Romani, N.; Aya, H.; Deguchi, M.; Ikehara, S.; Muramatsu, S.; Steinman, R. M. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med*. **1992**, *176*, 1693−1702.

(27) Fields, R. C.; Shimizu, K.; Mule, J. J. Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor immune responses in vitro and in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9482−9487.

DCM and plated at a density of 1×10^6 cells/mL per well in a 24-well plate, and cultured at 37 °C in 5% CO_2. On days 2 and 4, the medium was replenished by removing 50% of the DCM from the well and adding 1 mL of fresh DCM. On day 5 or 6, loosely adherent cells were harvested by gentle flushing of the wells, collected by centrifugation, and resuspended in RPMI-1640 complete at a density of $3-5 \times 10^6$ cells/mL. BMDCs were purified over a 14.5% (w/v) metrizamide (ICN Biomedicals) gradient, washed extensively, resuspended in DCM, and plated at a density of 1×10^6 cells/mL per well in a 24-well plate.

Generation of BMMΦs. Macrophages were grown as described by Racoosin and Swanson.[28] Briefly, bone marrow cells were flushed from the long bones of mice and were cultured in DMEM containing 20% fetal calf serum and 30% L-cell-conditioned media. On day 6, cells were harvested with PDE buffer, suspended in complete DMEM, and plated at a density of 1×10^6 cells/mL per well in a 24-well plate.

Characterization of BMDC and BMMΦ. The panel of primary antibodies used to characterize APCs consisted of anti-B7-2, anti-MHC II, anti-CD11c (BD PharMingen), and anti-DEC-205.[26,27] APCs were stained with 1 μg of primary antibody in PBS containing 2% BSA for 1 h at 4 °C, and then cells were washed and incubated with the corresponding secondary antibody (BD PharMingen) for 45 min at 4 °C. Cells were washed and analyzed using a FACSCalibur flow cytometer, and data were analyzed using CELLQuest software (Becton-Dickinson).

Antigen Presentation Assay. The antigen presentation assay was performed essentially as described in the previous reports with minor modifications.[12,13,21] On the day of the experiment, APCs were washed to remove serum-containing media and pulsed with liposomal antigen diluted in serum-free media for 3 h. The APCs were washed, chased in serum-containing media for 3 h, and then harvested by incubation with PDE buffer for 20 min at 4 °C. Cells were washed, fixed with 1% paraformaldehyde (Sigma), and treated with 0.2 M lysine (Sigma), followed by extensive washing. APCs were enumerated and plated at a density of 2×10^5 cells/well in a 96-well plate, followed by addition of 2×10^5 OVA-specific CD8 T-cells (CD8 OVA T1.3) to the well, and the plate was incubated for 24 h at 37 °C in 5% CO_2. The supernatant was collected, and IL-2 levels were measured by an enzyme-linked immunosorbent assay (ELISA) using anti-murine IL-2 capture and biotinylated detection antibodies (BD PharMingen). This IL-2 ELISA-based antigen presentation assay used for this study eliminates any direct IL-2 contribution from the APCs, which truly reports only the OVA peptide presentation by the APCs not the APC activation or costimulation.

Liposome Uptake Study. APCs were pulsed with liposomal HRP as described for OVA/LLO-liposomes with minor modifications. Briefly, APCs were pulsed with liposomal HRP in serum-free media for 3 h, washed extensively, and chased for 1 h in serum-containing medium. Cells were harvested with PDE buffer, washed, treated with lysis buffer (PBS Triton X-100, 2%, v/v), and kept frozen at −80 °C until they were analyzed. HRP uptake was assessed using the 1 Step Turbo TMB assay kit (TMB assay) (Pierce).

Trituration of Antigen. Protein was introduced directly into the cytosol by trituration to assess the efficiency of APCs in processing and presenting cytosolic antigen.[29,30] APCs were harvested and pelleted by centrifugation in a 15 mL conical tube; the supernatant was decanted, and the pellet was gently tapped. Then a 200 μL solution of serum-free media containing OVA (50 mg/mL) and HRP (20 mg/mL) was added to the pellet, immediately followed by pipeting the suspension for 30 cycles with a 200 μL tip, with the tip touching the bottom of the tube. Cells were washed extensively to remove unloaded protein and counted. Antigen presentation was assessed after a 2 h chase using the antigen presentation assay. Antigen uptake was assessed by monitoring HRP using the TMB assay.

Detection of MHC I−SIINFEKL Complexes. APCs were pulsed with the SIINFEKL peptide for 3 h at 4 °C, washed, and harvested with PDE buffer. The level of cell surface MHC I−SIINFEKL complexes was measured using a quantitative and a functional assay. Cell surface MHC I−SIINFEKL complexes were assessed by staining peptide-pulsed cells with the 25-D1.16 mAb, incubated with secondary antibody, and analyzed by flow cytometry. The functional effect of MHC I−SIINFEKL complexes was measured using the antigen presentation assay as described above.

Calculation of the Efficiency Ratio. The efficiency ratio was calculated by dividing antigen presentation by antigen uptake per cell.[31]

Results

Characterization of BMMΦs and BMDCs. To investigate and compare the efficiencies of antigen delivery by LLO-liposomes to two major APCs, BMMΦs and BMDCs, it is necessary to grow these two cell types in large numbers. The phenotypes of the cultured BMMΦs and BMDCs were characterized and compared by flow cytometry monitoring of differential expression of the cell type-dependent surface markers established for BMDCs: CD11c, DEC-205, MHC II, and B7.2.[26,27] BMDC culture used for this study exhibited a unimodal distribution for CD11c expression. BMDCs exhibited a bimodal distribution for DEC-205 and MHC II with one population expressing a high level of receptor and

(28) Racoosin, E. L.; Swanson, J. A. Macrophage colony-stimulating factor (rM-CSF) stimulates pinocytosis in bone marrow-derived macrophages. *J. Exp. Med.* **1989**, *170*, 1635−1648.

(29) Hollenbeck, P. J. Products of endocytosis and autophagy are retrieved from axons by regulated retrograde organelle transport. *J. Cell Biol.* **1993**, *121*, 305−315.

(30) Clarke, M. S.; McNeil, P. L. Syringe loading introduces macromolecules into living mammalian cell cytosol. *J. Cell Sci.* **1992**, *102* (Part 3), 533−541.

(31) Oh, Y. K.; Harding, C. V.; Swanson, J. A. The efficiency of antigen delivery from macrophage phagosomes into cytoplasm for MHC class I-restricted antigen presentation. *Vaccine* **1997**, *15*, 511−518.

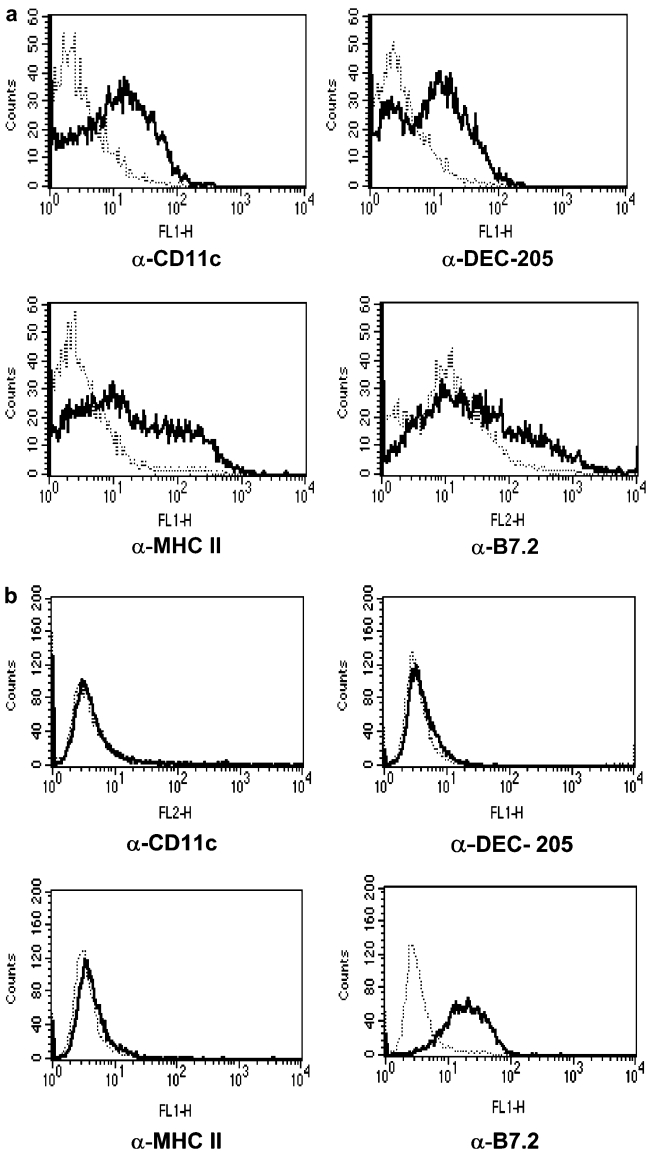

Figure 1. Flow cytometry analysis of APCs. (a) BMDCs and (b) BMMΦs were incubated with primary detection antibody to CD11C, DEC-205, MHC II, and B7.2. Cells were washed, treated with the corresponding secondary antibodies labeled with FITC or PE, and then analyzed by flow cytometry. The data shown with dotted lines represent nonspecific staining patterns by fluorophore-labeled secondary antibodies only, and the data shown with solid lines represent specific staining by primary detection antibodies followed by the secondary antibodies.

the other population with no detectable expression of receptor. BMDCs also appeared to exhibit a broad distribution for B7.2 with a major population expressing a low level of B7.2 and the other expressing a comparatively higher level of B7.2 (Figure 1a). In contrast, the cultured BMMΦs exhibited unimodal high levels of expression of B7.2 but no significant or detectable expression for CD11c, DEC-205, and MHC II (Figure 1b). The data suggested that BMDCs cultured for this study indeed possess DC characteristics but were a rather heterogeneous population in comparison, while

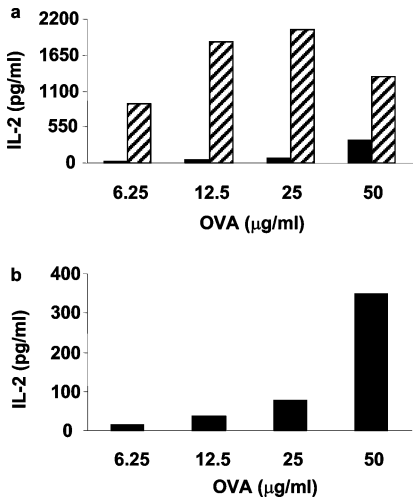

Figure 2. MHC class I-restricted presentation of liposomal antigen by APCs. BMDCs (solid bars) and BMMΦs (striped bars) were pulsed with liposomes containing OVA and LLO for 3 h. (a) After a 3 h chase, APCs were washed, fixed, and incubated with CD8 OVAT1.3 cells for 24 h. The supernatant was collected, and the amount of IL-2 was measured by an ELISA. (b) Close-up of antigen presentation by BMDCs (data shown in the solid bars in panel a). Data are representative of three independent experiments and are normalized for 1 $\times$ 10^6 cells ($n = 2$ for all BMMΦ data points; $n = 3$ for all BMDC data points, except $n = 2$ for an OVA concentration of 6.25 μg/mL).

the BMMΦs were a cell population that was quite homogeneous and free of contamination by other cells exhibiting BMDC characteristics.

LLO-Liposome-Mediated Antigen Presentation by BMDCs and BMMΦs. Utilizing the cultured BMDCs and BMMΦs, as characterized above, we first tested the overall efficiency of LLO-liposomes in mediating cytosolic delivery of antigen. To compare the relative ability of BMDCs and BMMΦs to present OVA encapsulated in LLO-liposomes, a well-established *in vitro* antigen presentation assay utilizing an OVA peptide-specific CD8 T-cell line (OVA T1.3) was used.[21] This T-cell line recognizes SIINFEKL, an eight-amino acid peptide fragment derived from OVA, complexed and presented on MHC I molecules (H-2K^b) by APCs. Recognition and engagement of the MHC I−SIINFEKL complex by the T-cell receptor leads to secretion of IL-2 by the OVA T1.3 cells. The quantity of IL-2 secreted in this assay is used as readout of the amount of OVA introduced into the cytosol of APCs and the extent of antigen presentation via the cytosolic pathway. BMMΦs exhibited a dose-dependent response for IL-2 production that saturated at 25 μg/mL liposomal OVA, beyond which the level of IL-2 detected in this assay either plateaued or was depressed (Figure 2a). BMDCs also exhibited a dose response, dependent on the concentration of liposomal OVA incubated with cells (Figure 2a,b). Both APCs when treated with a zero concentration of LLO-liposomal OVA produced no detectable level of IL-2 in this assay. Overall, at a given incubation concentration of LLO-liposomal OVA, BMMΦs exhibited

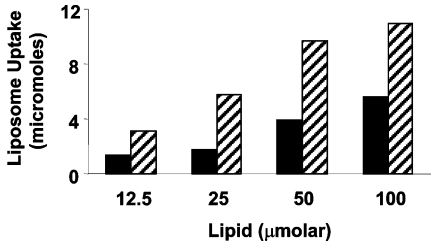

Figure 3. Liposomal uptake by APCs. BMDCs (solid bars) and BMMΦs (striped bars) were pulsed with liposomes containing HRP for 3 h. After a 3 h chase, APCs were extensively washed, treated with lysis buffer, and frozen at −80 °C, and the amount of cell-associated HRP was measured using the TMB assay. Data are normalized for 1×10^6 cells and are representative of two independent experiments.

more OVA peptide−MHC I presentation than BMDCs. Both APCs did not exhibit detectable antigen presentation when treated with OVA-containing, pH-sensitive liposomes without co-encapsulated LLO (data not shown). The data suggested that a difference in the efficiency of liposomal antigen presentation exists between the two APCs, and the difference must be due to the difference(s) in the relative efficiency of steps 1−4, outlined in the Introduction, in the two APCs.

Uptake of Liposomes by BMDCs and BMMΦs (Step 1). As the overall difference in the antigen presentation efficiency between BMDCs and BMMΦs was detected, we then investigated the mechanism for the difference by examining each stage in the multistep process of antigen presentation. We first hypothesized, as cellular uptake is typically dependent on cell type, that the foremost obvious step determining the overall difference in the antigen presentation is simply the differential capacity of binding and uptake of the liposomal formulation, which would dictate the overall extent of presentation at a given concentration of liposomal antigen incubated with a particular cell type. To test this hypothesis, BMDCs and BMMΦs were pulsed with liposomes containing HRP as a probe for the liposome-encapsulated protein. BMMΦs and BMDCs exhibited a dose-dependent binding and uptake as monitored by the amount of cell-associated liposomal HRP (Figure 3). BMMΦs had a higher level of HRP activity per cell than BMDCs at each liposomal HRP concentration incubated with the cells.

As we determined the overall presentation in the previous section and the uptake previously, the overall presentation per unit cellular uptake of antigen calculated as the ratio of IL-2 presentation to uptake was greater in BMMΦs (130−600 pg of IL2/μmol of uptake) than in BMDCs (30−90 pg of IL2/μmol of uptake). This suggested that the differences in the overall presentation of liposomal antigen could be partially explained but not entirely by the uptake differences between the BMMΦs and the BMDCs. This result points to the possibility that the differences in the efficiency of presentation of liposomal antigen are due to differences in steps 2−4.

Processing of Soluble Antigen in the Cytosol of the APCs (Step 3). Next, we tested the relative efficiency of

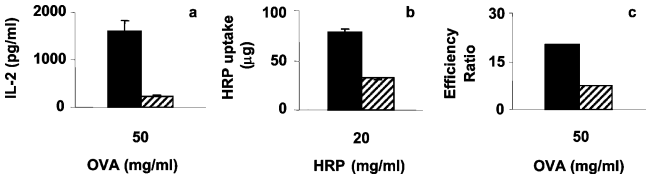

Figure 4. MHC class I presentation of trituration-loaded cytosolic antigen by BMDCs and BMMΦs. BMDCs (solid bars) and BMMΦs (striped bars) were loaded with OVA and HRP by trituration. (a) After a 2 h chase, APCs were washed, fixed, and incubated with CD8 OVAT1.3 for 24 h. The supernatant was collected, and the amount of IL-2 was measured by an ELISA. (b) Cells were collected, treated with lysis buffer, frozen at −80 °C, and tested for HRP activity using the TMB assay. (c) Antigen presentation was normalized by the amount of HRP loaded and expressed as an efficiency ratio. All data are normalized for 1×10^6 cells.

BMDCs and BMMΦs in processing and presenting protein antigen that is already present in the cytosol. To test this, OVA and HRP were co-introduced directly into the cytosol of the two APCs by temporary physical disruption of the plasma membrane integrity by trituration; this method has been previously employed successfully by others to introduce macromolecules into the cytosol for mechanistic manipulation of cytosolic events.[29,30] The extent of antigen presentation to T-cells after trituration loading of soluble OVA was higher in BMDCs than in BMMΦs (Figure 4a). The amount of protein loaded into cells during the trituration loading, as monitored by cell-loaded HRP after trituration, was also higher in BMDCs than in BMMΦs (Figure 4b). Therefore, the level of antigen presentation per cell had to be normalized by trituration loading of protein per cell to measure the efficiency per protein antigen introduced directly into the cytosol, and the data shown in Figure 4c suggest that BMDCs presented cytosolic antigen more efficiently than BMMΦs even after the normalization (Figure 4c).

Antigen Presentation by the Two APCs Loaded Externally with the SIINFEKL Peptide (Step 4). We then hypothesized that the differences in presentation between BMDCs and BMMΦs could be in part a function of the cell surface expression level of MHC I molecules. To determine the level of MHC I expression on the cell types, cells were pulsed with varying concentrations of the SIINFEKL peptide at 4 °C, limiting receptor turnover and internalization; afterward, the cells were stained with a monoclonal antibody (mAb 25-D1.16) that recognizes and binds to the MHC I−SIINFEKL complex.[24] The mean fluorescence intensity (MFI) of 25-D1.16 staining for BMDCs was approximately 3-fold higher than that for BMMΦs (Figure 5a) when cells were incubated with a saturating concentration of the SIINFEKL peptide, suggesting that the total number of SIINFEKL−MHC I complexes formed on the surface of BMDCs was greater than that found on BMMΦs. The data shown are from experiments using one saturating concentration of the SIINFEKL peptide, which was determined experimentally using escalating concentrations of peptides (data not shown).

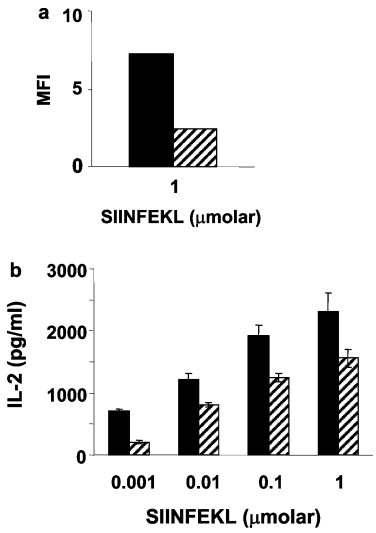

Figure 5. Detection of SIINFEKL−MHC I complexes on the surface of BMDCs and BMMΦs. BMDCs (solid bars) and BMMΦs (striped bars) were pulsed with the SIINFEKL peptide for 3 h at 4 °C. (a) BMDCs and BMMΦs were stained with primary antibody 25-D1.16, specific to the SIINFEKL−MHC I complex, washed, treated with secondary antibody conjugated to FITC, and analyzed by flow cytometry. The mean fluorescence intensities (MFIs) of 25-D1.16 staining for BMDCs and BMMΦs after incubation with a saturating concentration of the SIINFEKL peptide (1 μM) are shown. (b) BMDCs and BMMΦs were washed, fixed, and incubated with CD8 OVAT1.3 for 24 h. The supernatant was collected, and the amount of IL-2 was measured by an ELISA. Data are normalized for 1 $\times$ 10^6 cells.

We then assessed whether the differences in the number of SIINFEKL−MHC I complexes formed on the APC surface had a functional correlation to the level of IL-2 secretion by the OVA T1.3 cells that was used in the previous experiment described above. BMMΦs and BMDCs were pulsed with varying concentrations of the SIINFEKL peptide, as described above, and tested for their ability to present the antigenic peptide to T-cells using the antigen presentation assay. APCs exhibited a dose-dependent response over the range of SIINFEKL peptide concentrations that were used (Figure 5b). BMDCs exhibited a higher level of presentation of the peptide to T-cells than the BMMΦs, which was in agreement with the flow cytometry analysis using the 25-D1.16 mAb of SIINFEKL−MHC I complexes on the APCs.

Discussion

A series of complex steps are involved in the delivery of liposomal antigen to APCs and their presentation by the APCs. We assessed three key steps along the pathway in this investigation to determine the limiting steps that control the efficiency of LLO-liposomal antigen delivery and presentation by the two major APCs, DCs and MΦs.

First, we compared cultured murine BMDCs and BMMΦs in terms of the overall extent of antigen presentation using an assay that does not require a contribution from costimulatory molecules, thus allowing the comparison of the function of the relative number of SIINFEKL−MHC I complexes presented on BMDCs and BMMΦs after delivery.[21,22] The data showed that BMMΦs presented LLO-liposomal antigen more efficiently than BMDCs (Figure 2a). We then hypothesized that the higher overall level of antigen presentation by BMMΦs versus that of BMDCs is simply due to a greater uptake of liposomal antigen by BMMΦs. Gursel et al.[32] reported a similar trend in liposomal antigen uptake between spleen-derived DCs and MΦs using cationic liposomes. When the liposomal binding and uptake, step 1, was monitored, BMMΦs were indeed more efficient than BMDCs at "ingesting" liposomes via endocytosis/phagocytosis. When the presentation of antigen was normalized by the antigen uptake for the two cell types, BMMΦs still exhibited a higher efficiency ratio. This suggested the strong possibility that in addition to the higher level of antigen uptake by BMMΦs relative to BMDCs, the difference in efficiency of liposomal presentation lies in the differences in efficiency of steps 2−4 after the antigen uptake.

As it is difficult to determine directly the efficiency of LLO-mediated release of internalized liposomal antigen into the cytosol (step 2), we compared the downstream events by investigating the relative efficiency of BMMΦs and BMDCs in processing and presenting cytosolic antigen by directly loading the cytosol of these APCs by trituration with known amounts of antigen. The results showed that BMDCs were approximately 3-fold more efficient than BMMΦs when antigen was introduced by trituration (Figure 4c). Assuming that the other steps had the same efficiency in the two APCs, the result from the trituration experiment suggested that BMDCs would present liposomal antigen approximately 3-fold more efficiently than BMMΦs if equal amounts of antigen were delivered to the cytosol via the endosomal-to-cytosol pathway. This result pointed to two possibilities in steps 3 and 4: the antigen processing efficiency combined with loading of MHC I molecules with the processed peptides is more efficient in BMDCs, and/or the capacity, i.e., the number of peptide−MHC I molecules, is higher in BMDCs than in BMMΦs.

To determine the significance of each possibility, we tested the latter by monitoring how differences in the level of peptide-loadable MHC I expression between the two APCs affected the level of antigen presentation by these cells. The MHC I molecules on the two APCs were incubated with known concentrations of the SIINFEKL peptide, and the level of the SIINFEKL−MHC I complex that formed was assessed by two independent and complementary methods. It was observed that BMDCs exhibited a higher level of MHC I expression, which could be loaded with saturating concentrations of the peptide, than BMMΦs (Figure 5a). Accordingly, this higher level of expression was also translated to an increased efficiency of presentation of peptide

(32) Gursel, I.; Gursel, M.; Ishii, K. J.; Klinman, D. M. Sterically stabilized cationic liposomes improve the uptake and immunostimulatory activity of CpG oligonucleotides. *J. Immunol.* **2001**, *167*, 3324−3328.

to T-cells (Figure 5b). This suggests that BMDCs, in comparison with BMMΦs, exhibit a higher efficiency in presenting cytosolic antigen when the antigen is delivered into the cytosol, in part, due to a higher level of MHC I expression. This result, however, does not exclude the possibility that the capacity of antigen processing by proteasome into peptides and their transport to ER may also be greater in BMDCs than in BMMΦs. The peptide loading experiment in conjunction with the trituration study, in toto, supports the hypothesis that both steps 3 and 4 are more efficient in BMDCs than in BMMΦs.

In the experiments addressing the steps involved in antigen presentation, we investigated and compared steps 1, 3, and 4 in the two APCs. The results show that antigen uptake is more efficient in BMMΦs than in BMDCs, but that alone cannot explain the higher overall efficiency of antigen presentation of LLO-liposomal antigen by BMMΦs, as the antigen presentation per unit uptake by BMMΦs is still higher than that by BMDCs. As the antigen processing and presentation of antigenic protein once present in the cytosol, on the contrary, were determined in our study to be more efficient in BMDCs than in BMMΦs, the efficiency of antigen release from the internalized LLO-liposomes into the cytosol, step 2, must be significantly higher in BMMΦs than in BMDCs. Therefore, we conclude that the difference in the overall efficiency of liposomal antigen presentation is due to cell type-dependent differences in LLO-mediated release of antigen from the endosomal/lysosomal compartment, as well as due partially to the documented differences in the uptake of liposomal antigen by the two cell types.

This is the first report to compare the efficiency of delivery of protein using antigen entrapped in LLO-containing liposomes, head to head, in two different APCs. The cytosolic delivery was monitored by a well-established *in vitro* antigen presentation assay, and the two antigen-presenting cell types were derived from murine bone marrow progenitor cells. Our results indicate that LLO delivered via liposomes mediates antigen escape more efficiently in BMMΦs than in BMDCs. We propose that the difference is due to cell type-dependent activity of LLO, which in turn implies the possibility that *L. monocytogenes* may also escape more efficiently from the endosomal/lysosomal compartment of BMMΦs than from that of BMDCs.

The results reported here, however, should be taken with some caveats particularly because of the difficulty of culturing of DCs consistently and homogeneously despite spectacular advances in this field in recent years.[26] It is not straightforward to directly compare results from our study with those from other previous reports due to the variation in methods used to isolate and culture DCs. Variations in methods include species origin, precursor origin, length of culture, and cytokine stimulation. In some cases, these variables have been shown to have a dramatic effect on the functional activities of DC. Machy et al.[33] demonstrated that day 12 BMDCs, but not day 5 BMDCs, could present antigen entrapped in Fcγ receptor-targeted liposomes. In another study, Nair et al.[10] demonstrated a relatively efficient delivery of liposomal OVA to spleen-derived murine DCs. One possibility for the discrepancy between our data and their data, if it exists, is also that spleen-derived DCs may be functionally different from BMDCs. It is well-established that there are different subsets of DCs with different functions or levels of function.[17,34] On a case-by-case basis, it is difficult to directly compare results, which may necessitate comparison side by side or a better understanding of the effect of culture conditions on DC function.

With those caveats in mind, our results strongly support the hypothesis that the LLO activity in the endosomal compartment varies in different cell types, although the BMDCs used in our study may not be as homogeneous and as well-defined as one would hope. Our results also correlate with the observation that LM cells are predominantly retained within the phagosomal compartment of human DCs and seem not to invade the cytosol efficiently[35] and with the report that DCs located in the Peyer's Patch of rat intestine were a target of LM, but not permissive for replicative infection of LM.[36] One could argue that the lower LLO activity in the DC phagosome may be related to regulation of *Listeria* pathogenesis within the context of its interaction with the immune system. However, there are other reports describing the invasion of LM and LLO-expressing *E. coli* into the cytosol of BMDCs.[37,38] The idea of inefficient cytosolic invasion of LM into DCs is further complicated by the recent demonstration that CD11c+ DCs are required for generation of a CD8+ T-cell response toward LM.[39] As discussed above, it is entirely possible that LLO-mediated escape is dependent upon specific characteristics of the DC population,

(33) Machy, P.; Serre, K.; Leserman, L. Class I-restricted presentation of exogenous antigen acquired by Fcγ receptor-mediated endocytosis is regulated in dendritic cells. *Eur. J. Immunol.* **2000**, *30*, 848−857.

(34) Liu, Y. J. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell* **2001**, *106*, 259−262.

(35) Kolb-Maurer, A.; Gentschev, I.; Fries, H. W.; Fiedler, F.; Brocker, E. B.; Kampgen, E.; Goebel, W. *Listeria monocytogenes*-infected human dendritic cells, uptake and host cell response. *Infect. Immun.* **2000**, *68*, 3680−3688.

(36) Pron, B.; Boumaila, C.; Jaubert, F.; Berche, P.; Milon, G.; Geissmann, F.; Gaillard, J. L. Dendritic cells are early cellular targets of *Listeria monocytogenes* after intestinal delivery and are involved in bacterial spread in the host. *Cell. Microbiol.* **2001**, *3*, 331−340.

(37) Radford, K. J.; Higgins, D. E.; Pasquini, S.; Cheadle, E. J.; Carta, L.; Jackson, A. M.; Lemoine, N. R.; Vassaux, G. A recombinant *E. coli* vaccine to promote MHC class I-dependent antigen presentation, application to cancer immunotherapy. *Gene Ther.* **2002**, *9*, 1455−1463.

(38) Paschen, A.; Dittmar, K. E.; Grenningloh, R.; Rohde, M.; Schadendorf, D.; Domann, E.; Chakraborty, T.; Weiss, S. Human dendritic cells infected by *Listeria monocytogenes*, induction of maturation, requirements for phagolysosomal escape and antigen presentation capacity. *Eur. J. Immunol.* **2000**, *30*, 3447−3456.

(39) Jung, S.; Unutmaz, D.; Wong, P.; Sano, G.; De los Santos, K.; Sparwasser, T.; Wu, S.; Vuthoori, S.; Ko, K.; Zavala, F.; et al. In vivo depletion of CD11c$^+$ dendritic cells abrogates priming of CD8$^+$ T cells by exogenous cell-associated antigens. *Immunity* **2002**, *17*, 211−220.

which calls for further investigation using DC cultures under controlled conditions.

The potential sources of cell type-dependent variation of LLO activity include the composition of the endosomal/lysosomal compartment, such as the cholesterol content in the endosomal membrane, and its rate of acidification. Both pH-sensitive liposomes and LLO have optimal activity at the acidified pH of the early-to-late lysosomal compartment.[16,40,41] The composition of cholesterol of the endosomal/lysosomal compartment is also not well defined and could vary depending on cell type, which may affect the activity of LLO.[42,43]

Future studies correlating LLO-dependent delivery with endolysosomal characteristics in different APCs and cell types may yield clues about the subsets of cells preferred by LM if one adopts a simplistic view of LLO being the primary factor in the endosomal escape of LM. The LLO-liposomal system will be beneficial, in addition to being a viable vaccine formulation for CTL generation, by providing a means of studying the influence and mechanism of LLO-mediated endosomal disruption; LLO can be studied alone or in combination with other pathogenic determinants, such as phospholipases, that may also facilitate the escape of LM.

Acknowledgment. This work was supported by University of Michigan NIH Pharmacological Sciences Training Program Grant GM07767, Rackham Discretionary Funds (to E.M.S.), NIH Grants R21AI42657, R01AI47173, and R01AI58080 (to K.-D.L.), and an Upjohn grant to the College of Pharmacy (to M.M.). We thank Dr. Ralph Steinman (Rockefeller University), Drs. James Mule and Blake Roessler (University of Michigan), and all members of the Lee laboratory, especially Claire Reinhardt, for their help in DC culture.

MP049896V

(40) Straubinger, R. M. pH-sensitive liposomes for delivery of macromolecules into cytoplasm of cultured cells. *Methods Enzymol*. **1993**, *221*, 361−376.

(41) Glomski, I. J.; Gedde, M. M.; Tsang, A. W.; Swanson, J. A.; Portnoy, D. A. The *Listeria monocytogenes* hemolysin has an acidic pH optimum to compartmentalize activity and prevent damage to infected host cells. *J. Cell Biol.* **2002**, *156*, 1029−1038.

(42) Maxfield, F. R.; Wustner, D. Intracellular cholesterol transport. *J. Clin. Invest.* **2002**, *110*, 891−898.

(43) Rossjohn, J.; Feil, S. C.; McKinstry, W. J.; Tweten, R. K.; Parker, M. W. Structure of a cholesterol-binding, thiol-activated cytolysin and a model of its membrane form. *Cell* **1997**, *89*, 685−692.