Enhanced antigen presentation and CTL activity by transduction of mature rather than immature dendritic cells with octaarginine-modified liposomes

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Abstract

To improve uptake and cross-presentation of exogenous antigens (Ag) by dendritic cells (DCs), octaarginine-modified liposomes (R8-Lip) were used as a novel strategy for protein-Ag transduction. Immature DCs endocytose macromolecules efficiently. While mature DCs lose their ability to capture Ag, but have an increased capacity for T-cell activation. Thus Ag-transduction has been performed mostly in immature DCs. In the present study, R8-Lip were efficiently taken up by both immature and mature DCs. DCs transduced after maturation were highly efficient at cross-presentation of Ag and induced higher cytotoxic T-lymphocytes (CTL) activity than were DCs transduced before maturation. The mechanism of Ag presentation involved the escape of R8-Lip from endosomes to cytosol, which require the acidic environment. The Ag released was then processed by a proteasome-dependent pathway. This novel transduction approach is clinically applicable, easy to perform, and has more practical advantages than current protein transduction methods.

Keywords: Dendritic cells/ Protein transduction/ Octaarginine-modified liposomes/ Antigen presentation/ Cytotoxic T lymphocytes

INTRODUCTION

The unique ability of dendritic cells (DCs) to take up, process and present antigens (Ag), and to activate naïve CD4+ and CD8+ T cells raises the possibility of using DCs as "nature vaccines" [1, 2]. So far, the efficiency of Ag-loaded DCs to induce immunity against tumors and viral infectious diseases has been tested in a number of pre-clinical trials [1-3]. Although DCs-based immunotherapy presents a promising approach, optimization of DCs-vaccination protocols is still needed to enhance the success of future clinical trials [3]. In most trials, Ag was loaded into DCs *in vitro* and the cells were then administered to patients.

One of limiting steps in dendritic cells (DCs)-based vaccination is the maturation stage of the cells. Immature DCs specialize in Ag capture, whereas mDCs have a low capacity for Ag uptake capacity, but an increased ability to stimulate T-cells [4, 5]. Because the Ag uptake by DCs decreases as the cells mature from iDCs to mDCs, most groups have used iDCs to promote Ag capture and uptake [6]. To facilitate the Ag uptake by DCs, arginine-rich cell-penetrating peptides (CPPs) including HIV-*TAT*, poly-arginine and penetratin (*Int*) derived from Antennapedia has been applied recently [7-10]. Kim *et al.* were the first to demonstrate that Ag-specific cytotoxic T lymphocytes (CTLs) are generated *in vivo* by immunizing mice with DCs that had been exposed to protein-*TAT* conjugates [7]. Since then various research groups, including our own, have applied CPP transduction to DCs as a method of stimulating Ag-specific T cells. Many studies reported that mice immunized with DCs transduced with recombinant CPP-Ag protein suppressed the tumor growth significantly [8-15]. Apart from the recombinant fusion protein, synthetic polypeptide containing CPP tandemly linked to peptide epitopes is another striking approach [14]. Although recombinant

proteins or peptides containing CPPs are attractive prospects, a specific construct needs to be made. In addition, peptide synthesis and construction/ purification of such fusion proteins are tedious and complex work.

Recently our group has constructed a simple and efficient vaccine delivery system so called “ocataarginine-modified liposomes” (R8-Lip), in which the encapsulated Ag can be delivered, processed and presented to specific T-cells efficiently [16]. We have found that R8-Lip with Ag encapsulated induced a strong anti-tumor effect in a mouse model. In the present study, we demonstrate that R8-Lip can be taken up by DCs efficiently, regardless of DCs maturation stage. The escape of R8-Lip from endosomes to the cytosol was highly efficient in mDCs and the released Ag were processed via the proteasome-dependent pathway. Interestingly, we found that when the order of Ag-loading and DCs maturation is reversed; that is DCs maturation is induced first, followed by the Ag-transduction by R8-Lip, the pMHC class I presentation and CTL activities have markely improved. These findings would be an advantage for further development of DCs-based vaccination.

Materials and Methods

Materials

Dioleoyl phosphatidylethanolamine (DOPE) and egg phosphatidylcholine (EPC) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesteryl hemisuccinate (CHEMS) was obtained from SIGMA-Aldrich Co. (St. Louis, MO). Sulforhodamine B, and Lysotracker Red were purchased from Molecular Probes (Eugene, OR). Stearylated-octaarginine (STR-R8) was synthesized as described previously [17]. Synthetic oligodeoxynucleotides (ODN) containing CpG motifs (CpG 1668; 5'-TCCATGACGTTCCTGATGCT-3') were purchased from Hokkaido System Science (Sapporo, Japan). Other chemicals were purchased from Wako Chemicals (Osaka, Japan)

Preparation of R8-modified liposomes encapsulating OVA antigens

Liposomes were comprised of DOPE, CHEMS, and EPC in a molar ratio of 7.5:1:1.5. The desired amount of each lipid in chloroform was dried under a nitrogen stream. The lipid was then hydrated by adding 1 ml 10 mM Hepes buffer, containing OVA (5 mg OVA), and incubating the mixture for 10 min at room temperature with gentle shaking. Sulforhodamine B was used as an aqueous phase marker, when required. Preparations were then sonicated and subjected to six freeze-thaw cycles. The vesicle diameter was controlled by sequentially extruding the preparation through polycarbonate membrane filters with a pore size of 400 nm. Then, any OVA that was not associated with the liposomes was removed by ultracentrifugation at 80,000 x g (Himac CS150 GX, Hitachi) for 30 min at 4 ^{o}C. The final liposome pellets were resuspended in 1 ml Hepes buffer. Surface modification of the resulting liposomes with polyarginine peptides was

accomplished by incubating the liposomes with an aqueous solution of STR-R8 (7.5 mol% total lipids) for 30 min at room temperature.

The diameter and zeta-potential of liposomes were measured using an electrophoretic light-scattering spectrophotometer (Zetasizer Nanoseries, Malvern Instruments, Malvern UK). The concentration of phospholipids in the liposome suspension was measured by the cholineoxidase method using Phospholipid C-Test (Wako Pure Chemical Industries, Osaka, Japan). The amount of entrapped OVA was quantified after lipid removal, according to the method of Peterson [18].

Dendritic cells generation

Bone marrow-derived DCs were obtained as described previously [19]. Briefly, bone marrow was flushed from femurs and tibias of C57BL/6 mice. After lysing the erythrocytes, cells were cultured in 24-well plates (10^6 cells/well) in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 50 mM 2-mercaptoethanol, 25 mM Hepes, 50 IU of penicillin and 50 μg of streptomycin (PS) per ml in the presence of 500 U/ml of recombinant murine granulocyte macrophage colony stimulating factor (GM-CSF). The floating cells were replated on day 2. On days 3 and 4, nonadherent cells (newly formed granulocytes) were gently removed from the cultures, and fresh medium plus GM-CSF was added. On day 7, DCs were harvested and the cell yield was analyzed using Trypan blue staining.

Kinetics of liposome-uptake by dendritic cells

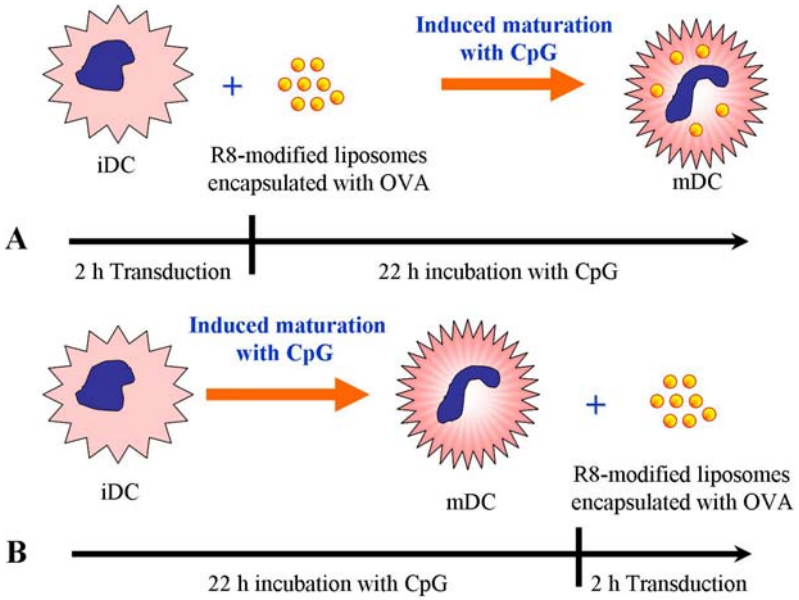
The kinetics of endocytosis of non-modified liposomes and octaarginine-modified liposomes (R8-Lip) by DCs were quantitatively determined by flow cytometry.

DCs (5 x 10^5) harvested on day 7 were cultured in 1 ml of RPMI 1640 medium supplemented with 10% FCS, PS and GM-CSF. Differentiation of iDCs into mDCs was induced by adding 1 μg/ml CpG-ODN to the cell cultures. The cells were harvested at various time points and washed twice with ice-cold PBS. Next, the cells were suspended in 0.5 ml serum-free medium containing sulforhodamine labeled-liposomes or sulforhodamine labeled-R8-Lip (final concentration, 0.1 mM lipids) for 1 h at 37 ^{o}C. At the end of the incubation, the medium was removed, and the cells were washed three times with FACS buffer (PBS containing 0.5% BSA and 0.1% NaN_3). The cells were then suspended in 0.5 ml FACS buffer, and, after passing through a nylon mesh, were analyzed by flow cytometry using a FACSort (Becton Dickinson) equipped with Cell Quest Software (BD Biosciences).

Transduction of iDCs and mDCs with liposomes

The procedure for transducing R8-Lip-OVA into either iDCs or mDCs is shown in schematic form in Fig. 1.

Figure 1



After seven days of culture, DCs were harvested and then resuspended at a concentration of 1.2 x 10^6 cells/ 0.5 ml in serum free media. The cells were seeded into 24-well plates. For iDCs transduction (transduced 2 h → mat 22 h), 2 μg of free OVA, liposomes or R8-Lip, both encapsulating similar amounts of OVA, were added. The suspension was mixed well, and incubated at 37°C. After 2 h of incubation, the cells were washed twice with PBS. Then, two ml of complete media were added. Finally, transduced iDCs were differentiated into mDCs by treatment with CpG-ODN (1 μg/ml) for 22 h. For mDCs transduction (mat 22 h → transduced 2 h), DCs maturation first was induced with CpG-ODN (1 μg/ml) for 22 h in complete media. After differentiation, the mDCs were collected and resuspended in 500 μl serum-free media. Then, the mDCs were transduced with 2 μg free OVA, liposomes or R8-Lip, encapsulating similar amount of OVA. After 2 h of incubation, the cells were washed twice with PBS. The effect of the Ag-loading dose into DCs was also examined by transduction DCs with R8-Lip-OVA at lipid concentrations of 0.025, 0.05, 0.075 and 0.1 mM (~ca. 0.3, 1.3, 2, and 2.7 μg OVA respectively).

Antigen presentation assays

The level of OVA-derived peptide/MHC class I complexes (pMHC) expressed on DCs was determined by measuring of *lacZ* activity induced in B3Z $CD8^+$ T-cell hybridomas, as described elsewhere with some modifications [20]. Briefly, transduced DCs (a total of 2 x 10^5 cells/well) were co-incubated with B3Z T cells (1 x 10^5 cells/well) in flat bottom 96-well plate for 15 h. After incubation, cultures were washed once with 100 μl PBS and *lacZ* activity was measured in the lysates by addition of 100 μl of 5 mM chromogenic *lacZ* substrate--chlorophenol red β-D-galactopyranoside (CPRG, Roche, IN) in PBS containing 0.5% NP-40. The *lacZ* enzyme was quantified by measuring, the reaction product after a 4 h incubation at 37^{o}C, chlorophenol red. The amount of pMHC presented by DCs was calculated by comparing the UV absorption (595 nm) values of OVA-treated and untreated DCs cultures.

Immunizations and CTL assays

Purified DC, both immature and mature cells, were pulsed with R8-Lip-OVA as described above. The resulting DCs (7.5 x 10^5) were intradermally injected into four C57BL/6 mice at day 0. At day 7, the mice were given a second injection, using the same protocol. Then, on day 14, spleens were harvested and re-stimulated with an OVA-expressing K^b-restricted tumor cell line, EG.7. Before use, the EG.7 cells were pretreated with mitomycin C (50 μg/ml for 30 min) and were then added to the isolated splenocytes at a ratio of 10:1 (splenocytes:EG.7). Five days later, splenocytes were harvested and used as effector cells in the CTL assays.

CTL assays were performed using a standard chromium-release assay. ^{51}Cr-labeled EG.7 cells were co-cultured with variable numbers of splenocytes (effector cells) in 96-well V-bottom culture plates at 37^{o}C for 4 h. The ^{51}Cr activity released into the culture supernatant was measured. Specific cytotoxicity was calculated as follows: [(experimental ^{51}Cr release – spontaneous ^{51}Cr release)/(maximum ^{51}Cr release – spontaneous ^{51}Cr release)] x 100%. Spontaneous and maximum release were determined by the activity in wells containing medium alone or after lysis with 1 N HCl, respectively.

Inhibition studies

For inhibition studies, iDCs or mDCs were preincubated for 30 min with various inhibitors: amiloride (0.5 and 1.0 mM) (Sigma), chloroquin (5 and 20 μM) (Wako Pure Chemicals), and ammonium chloride (50 and 100 mM) (Wako Pure Chemicals). After preincubation, R8-Lip-OVA were added to the cell cultures at final lipid concentration of 0.075 mM (ca~ 2 μg OVA). After an additional 2 h incubation at 37^{o}C, the cells were washed twice with ice-cold PBS and then co-cultured with the B3Z T cells, as described above.

To evaluate whether the proteasomal complex or endosomal/lysosomal proteases were required for Ag processing, DCs were pre-incubated with 1, 10, 25, and 50 μM of lactacystin (a specific inhibitor of the 26S proteasome) or with 50, 100, 250, and 500 μM of leupeptin (a protease inhibitor) for 30 min at 37^{o}C. Then R8-Lip-OVA (0.075 mM lipid concentration) were added to the cultures and the cells were incubated in the presence of inhibitors for an additional 2 h. The DCs were then washed with PBS and added to B3Z T cells.

Intracellular localization of antigens

Cellular uptake and intracellular localization of OVA encapsulated into R8-Lip were examined using confocal laser scanning microscopy (CLSM). OVA was conjugated with fluorescein isothiocyanate (FITC, Sigma). Labeled OVA was then loaded into R8-Lip, as described above. Immature DCs and mDCs, 2 x 10^5 cells/0.5 ml, were resuspended in serum-free RPMI 1640 medium. Viable cells were then incubated for 2 h at 37^{o}C with R8-Lip loaded with OVA-FITC (final concentration of 0.075 mM lipid) in polypropylene tubes. Thirty minutes prior to visualization, 75 nM LysoTracker Red solution—an endosome and lysosome marker—was added. After incubation, the cells were washed three times with ice-cold PBS. The cells were then resuspended in 0.5 ml PBS and put into 35-mm glass-base dishes (Iwaki, Chiba, Japan). Finally, the cells were directly examined with a CLSM (LSM510 meta, Carl Zeiss) equipped with a 488-nm argon, and 543 HeNe laser. The laser power and photomultiplier settings were kept constant for all samples.

Results

Octaarginine liposomal formulation loaded with OVA

Octaarginine-modified liposomes encapsulating OVA (R8-Lip-OVA) were prepared according to the post insertion technique. The non-modified liposomes were first prepared and then these liposomes were incubated with stearylated octaarginine (STR-R8). During the incubation, the membrane-permeable peptides, R8, attach to the liposome surface.

Table 1: Physical properties of Non-modified and Octaarginine (R8) modified liposomes encapsulating ovalbumin (OVA)

Liposome Formulations [a]	Particle Size (nm)	P.D. [b]	ζ-potential (mV)
Non-modified liposomes	160 ± 11	0.18 ± 0.07	-52.7 ± 9.7
R8-modified liposomes	196 ± 21	0.21 ± 0.04	50.7 ± 6.3

Values are mean ± S.D. (n = 6).

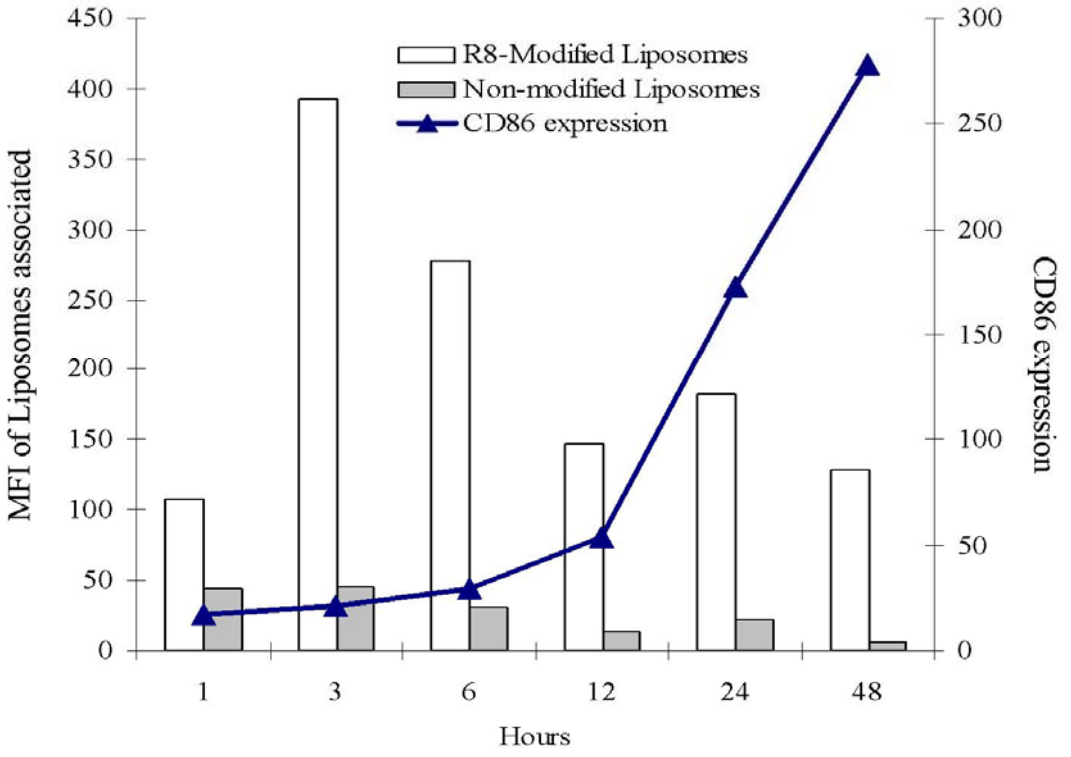
Table 1 summarises the physical properties of the non-modified liposomes and R8-Lip-OVA, both encapsulating OVA. The presence of arginine octamers on the liposome surface resulted in a slight increase in particle size (~ca. 200 nm). As expected, non-modified liposomes were negatively charged, as indicated by their zeta potential, whereas R8-Lip were positively charged.

Kinetic of liposomes uptake and DCs maturation

The relationship between liposomal uptake and maturation stage of DCs was determined by measuring the amount of fluorescence incorporated into DCs after 1 h of incubation with rhodamine-labeled liposomes. In addition, maturation was induced by treatment of DCs with CpG-ODN, and the level of CD86 expression was used as a

maturation indicator. As shown in Fig. 2, R8-Lip treated DCs showed higher levels of fluorescence than DCs treated with non-modified liposomes at all time points.

Figure 2



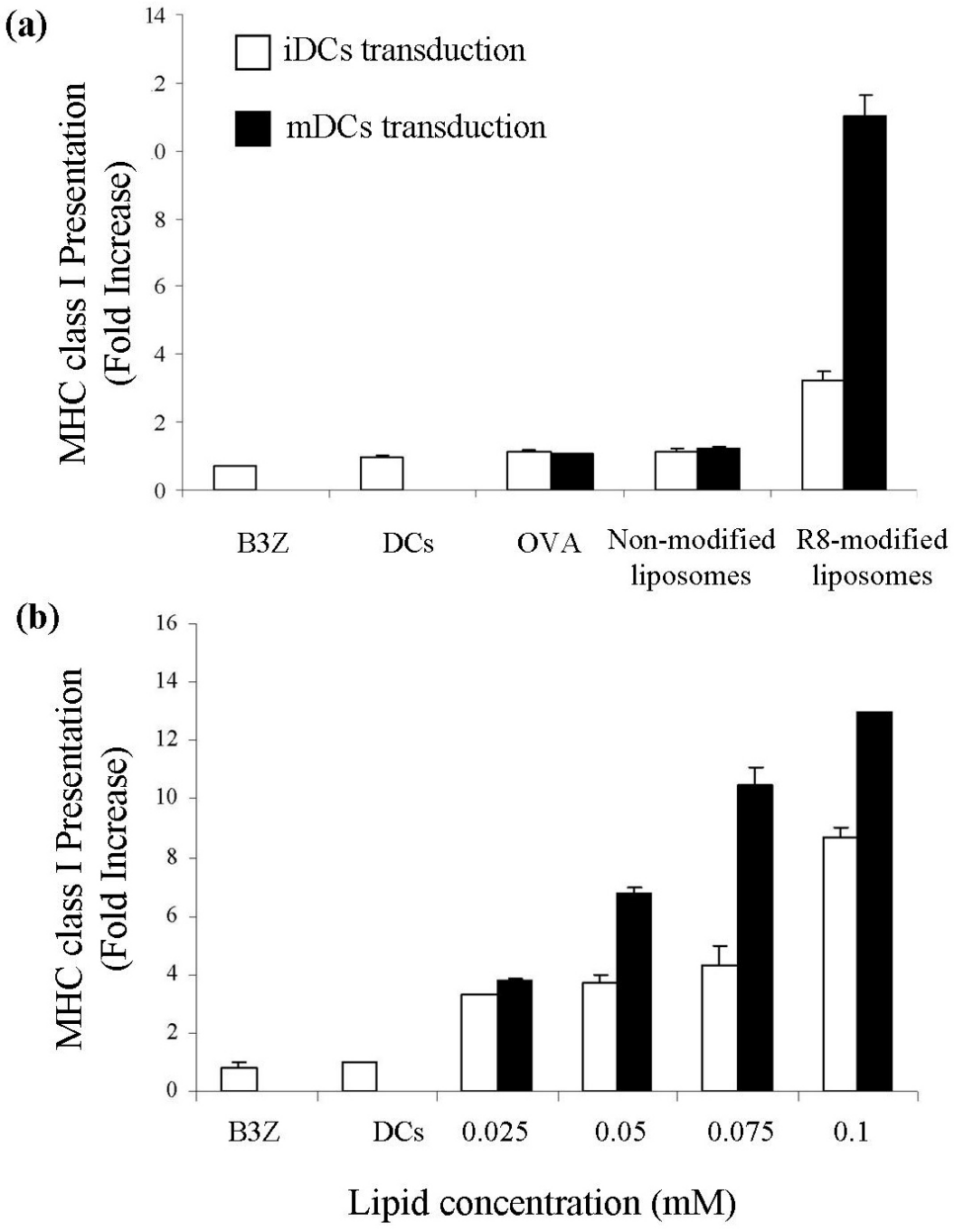
This result indicates that liposomal uptake was enhanced by R8-surface modification. Except for DCs harvested at 1 h, R8-Lip uptake decreased over time, but was still much greater than that of the non-modified liposomes. Maturation of DCs was verified by upregulation of CD86 expression. CD86 was sharply increased after 12 h of incubation with CpG-ODN.

Transduced mature DCs are more efficient at pMHC presentation to T cells than immature DCs

To compare the antigen presentation capacity of transduced iDCs and mDCs, DCs were cultured and pulsed with free OVA, non-modified liposomes loaded with OVA or R8-Lip-OVA. As shown in Fig. 3a, only DCs transduced with R8-Lip-OVA,

either before maturation (transduced 2h → mat 22 h) or after maturation (mat 22 h → transduced 2 h), were able to present pMHC efficiently to B3Z T cells. Yet the mDCs pulsed with R8-Lip-OVA demonstrated a considerably greater capacity for pMHC presentation than iDCs transduction.

Figure 3



DCs, pulsed with OVA solution (2 μg) and non-modified liposomes encapsulating the similar amount of OVA could not present Ag to specific T cells. There was no auto-activation of B3Z T hybridoma cells, as evidenced by the similar absorbance values for the B3Z T cells and untreated DCs (i.e., negative control).

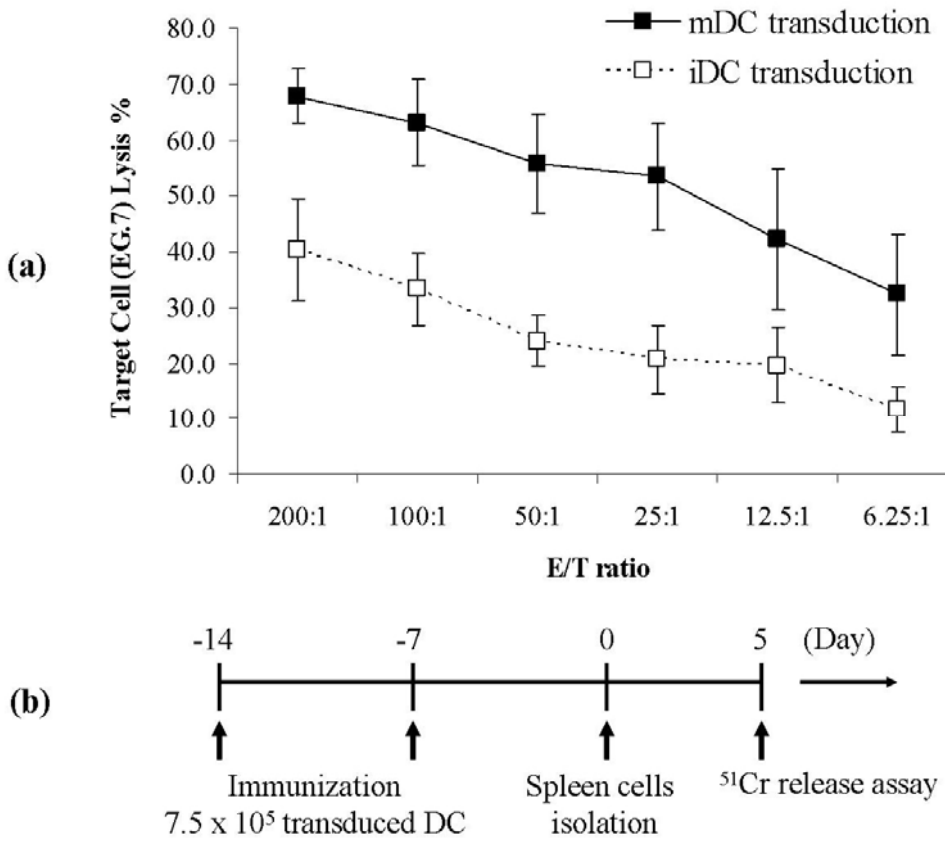
To investigate the influence of Ag concentration loaded into DCs on the efficiency of pMHC class I presentation, iDCs and mDCs were cultured and pulsed with different concentration of R8-Lip-OVA, i.e. 0.025, 0.05, 0.075, and 0.1 mM lipid concentrations. The ability of the pulsed DCs to activate B3Z T cells was then determined. For mDCs transduction (mat 22 h → transduced 2 h), activation of B3Z T cells was highly dependent on the R8-Lip-OVA concentration (Fig. 3b). Increasing the R8-Lip-OVA concentration from 0.025 to 0.1 mM, resulted in a 3.7-fold increase in pMHC presentation efficiency. However, for iDCs transduction (transduced 2 h → mat 22 h), there was no much difference in pMHC presentation efficiency observed for 0.025 to 0.075 mM of R8-Lip-OVA. We also found that the much larger amount of free OVA (over 1 mg/ml range) has to be used for the similar efficiency of pMHC presentation (data not shown).

Priming of cytotoxic lymphocytes after administration of R8-Lip transduced DCs

To test whether iDCs and mDCs treated with R8-Lip-OVA were able to stimulate OVA-specific CTL responses, DCs were incubated with R8-Lip-OVA (5 μg OVA) and cells were then administered intradermally to naïve mice. The mice were given a booster injection of pulsed DCs one week after the first immunization. As shown in Fig. 4, an OVA-specific CTL activity of splenocytes from mice immunized

with transduced mDCs was nearly twice that observed for splenocytes from mice immunized with transduced iDCs.

Figure 4



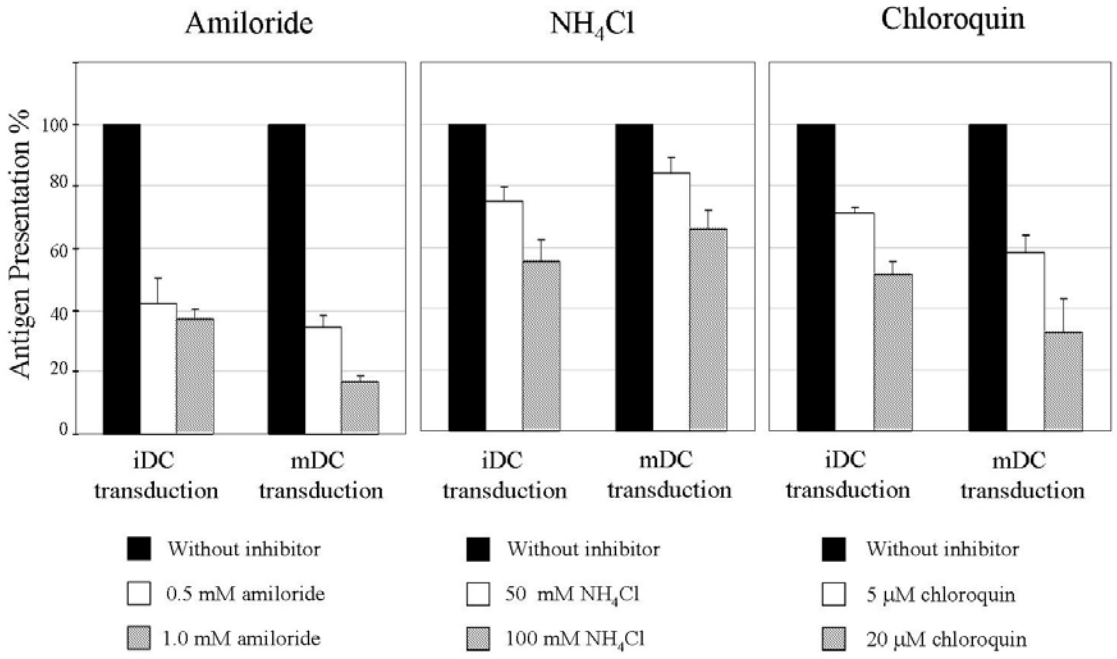
These implied that transduction with R8-Lip in mature stage was more efficiency than that in immature cells.

The role of macropinocytois and endosomes/lysosomes in the uptake and presentation of R8-Lip-OVA

To ascertain if the uptake of R8-Lip-OVA by DCs occurs via macropinocytosis, an inhibitor of Na^+/H^+ exchange—amiloride—was used in the Ag-presentation assays. No death or toxicity was observed after simultaneous treatment with the inhibitors and

R8-Lip (data not shown). Amiloride (1.0 mM) inhibited the presentation of pMHC by 65% and 85% for transduced iDCs and mDCs, respectively (Fig. 5).

Figure 5

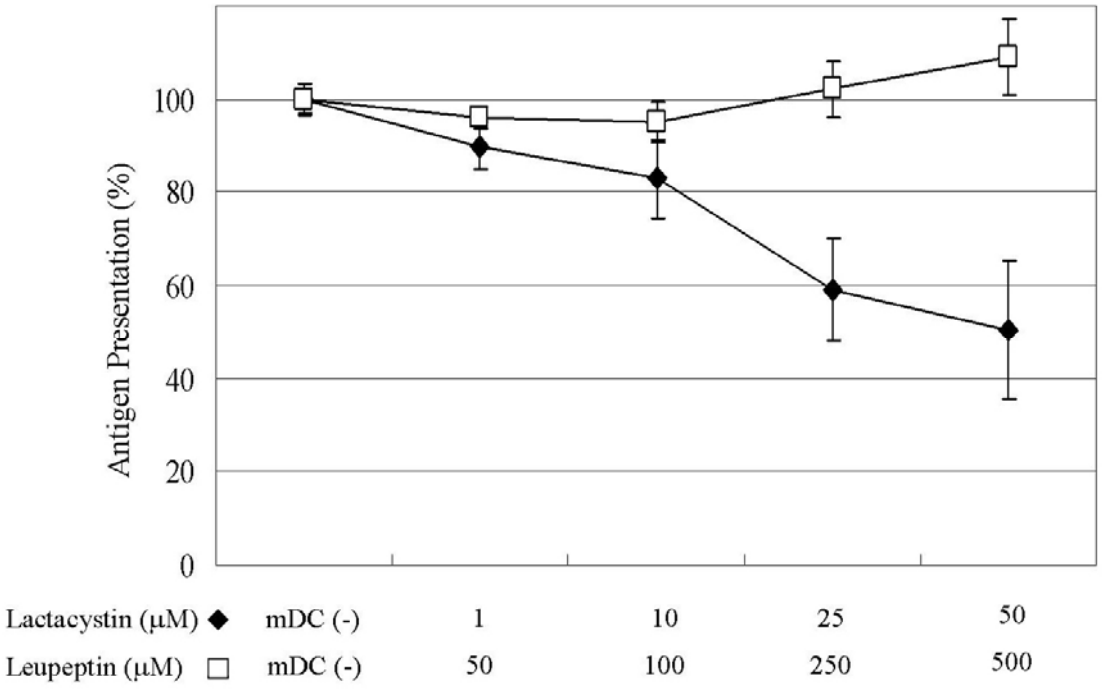


The inhibition of pMHC presentation by amiloride suggests that uptake of R8-Lip-OVA involves macropinocytosis. The role of the endosome in Ag presentation was investigated using NH_4Cl and chloroquin, which increase endosomal pH, preventing acidification of endosomes. Addition of NH_4Cl (100 mM) to pulsed iDCs and mDCs, inhibited pMHC presentation to B3Z T cells by 45% and 35%, respectively (Fig. 5). A similar result was obtained for the chloroquin inhibition studies. These results suggested that at less acidic endosomal pH of DCs the Ag encapsulating in R8-Lip has lower ability to access the MHC class I machineris.

MHC class I presentation after R8-Lip-OVA internalization is lactacystin sensitive

As shown in Fig. 6, Ag-presentation by mDCs transduced with R8-Lip-OVA was somewhat inhibited by low concentration of lactacystin (1 and 10 μM).

Figure 6

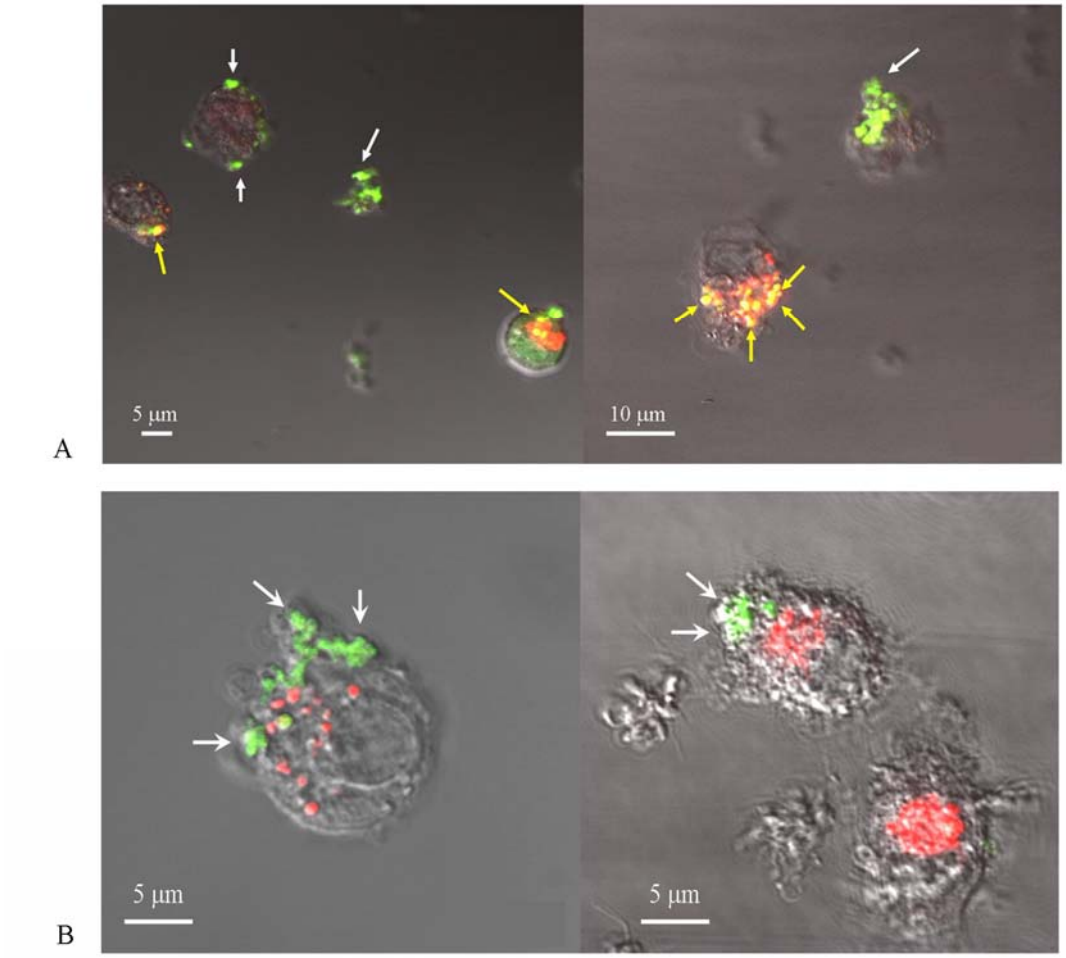


The pMHC presentation was markedly inhibited at the higher concentration of lactacystin (by 40% and 50% at 25 and 50 μM, respectively). However, pre-treatment of mDCs with leupeptin, an inhibitor of the endosomal protease cathepsin B, and of serine and cysteine proteases, had no effect on the presentation of pMHC to B3Z T cells. These results suggest that pMHC class I presentation of Ag delivered by R8-Lip would occur mainly via the conventional MHC class I presentation pathway.

Compartmentalization of R8-Lip loaded with fluorescently labeled OVA in DCs

To visualize the intracellular localization of OVA in DCs, R8-Lip encapsulating fluorescein-labeled OVA were prepared. The potentials of iDCs and mDCs to take up R8-Lip containing FITC-OVA were then determined by confocal microscopy. The fate of labeled-OVA in iDCs and mDCs were visualized by staining DCs with LysoTracker Red, which accumulates in endosomes and lysosomes.

Figure 7



As shown in Fig. 7A, most of the labeled-OVA (green fluorescent) in iDCs was co-localized with LysoTracker (as indicated by yellow arrows). Only some of the labeled-OVA was observed at the cellular membrane and intracellularly, but not co-localized with LysoTracker staining (as indicated by white arrows). In contrast, Lysotracker and labeled-OVA were clearly distinguished at unique intracellular locations (white arrows) in mDCs (Fig. 7B). Thus, these results suggest that the OVA encapsulated in R8-Lip escapes from the endosomes and lysosomes of DCs, particularly in the case of mDCs.

Discussion

A critical process in the activation of CTLs is the efficiency of DCs in picking up and processing Ag into the MHC class I presentation pathway. This pathway is currently called "cross-presentation" pathway [4, 21]. In an attempt to enhance the cellular uptake and promote the cross-presentation pathway in DCs, many investigators perused the recombinant fusion protein or synthetic peptides which contain CPPs tandemly linked to the Ag [7-14]. However the construction of such a recombinant proteins/ peptides are specific for a single Ag, limiting the usefulness of this technology. Recently, we have reported the novel vaccine delivery system, R8-Lip, which can induce immunity against tumor model successfully [16]. Liposomes can encapsulate various kinds of proteins/ peptides Ag, regardless of their hydrophobicity. In addition, the arginine octamer peptides anchored to the surface of liposomes enhance the uptake and cross-presentation of Ag. The simplicity, versatility and advantage of R8-Lip has prompted us to investigate their transduction efficiency in DCs.

Immature DCs rapidly consume Ag. So the use of DCs vaccine has generally been through transduction/ electroporation or transfection of Ag in immature DCs followed by an induction of DCs maturation [4, 21]. Van Tendeloo *et al*. showed that the most potent CTL activation was achieved when mRNA loading by electroporation or lipofection was performed prior to maturation of monocyte-derived DCs [22]. The authors attributed the reduced capacity to stimulate T-cells by DCs electroporated after maturation to lower transduction efficiency. However, recent studies have demonstrated that RNA transfer by electroporation into DCs, was high, regardless of maturation stage [23-26]. In addition, electroporation of DCs after maturation appears to be preferable for DCs vaccines because electroporation of such those DCs are immunologically superior

for CTL induction [24, 26]. In the present study, we also found that transduction of mDCs with R8-Lip was preferrential over transduction of iDCs.

The transduced iDCs and mDCs exhibited similar immunophenotypes and maturation potentials, regardless of when they were transduced with R8-Lip-OVA (data not shown). DCs transduced with R8-Lip-OVA after maturation were by far superior in presenting pMHC as compared to those transduced in the immature stage (Fig. 3). In addition, a correlation between *in vitro* pMHC presentation capacity of DCs and cytolytic activity against specific target cells, EG.7, was observed in this study. Mice injected intradermally with R8-Lip-OVA transduced mDCS (mat 22 h → transduced 2 h) revealed relatively high CTL activity (Fig. 4), consistent with the high *in vitro* pMHC presentation. Our results were in agreement with many studies, which reported that transduction of mDCs with recombinant fusion proteins containing HIV-*Tat* was preferential over transduction of iDCs [8, 12, 13]. The longevity of pMHC on the surface of mDCs might be an advantage of pulsing Ag to DCs after their maturation [27, 28]. The pMHC on the surface of mDCs is more stable and there is less membrane turnover compared with iDCs [27-29]. It is possible that transduction of mDCs with recombinant CPPs or R8-Lip allows DCs to process and present internalized Ag to T-cells using newly synthesized MHC class I molecules. As a consequence, transduction of mDCs with R8-Lip-OVA showed higher pMHC class I presentation and CTL activity.

To a large extent, the efficiency of pMHC presentation is dependent on the concentration of pMHC complexes on the DCs surface [4, 21]. A presentation of pMHC derived from exogenous protein is inefficient, since it requires relatively high concentration of proteins Ag – in the mg/ml range [21]. However, R8-Lip-OVA were

capable of inducing MHC class I presentation of OVA epitope at a relatively low dose (2 μg). The efficiency of pMHC presentation was also greater with the higher concentration of R8-Lip-OVA (Fig. 3b), but still required a small quantity of Ag (in a μg/ml range). This novel transduction method would be an advantage if multiple Ag, such as various CTL epitopes, coule be simultaneously codelivered and cross-presented efficiently even at low concentration.

The intracellular trafficking path of Ag determines the resulting presentation modality. In agreement with our previous studies, treatment of either iDCs or mDCs with amiloride (a macropinocytotic inhibitor) reduced pMHC presentation significantly [16]. Pouniotis *et al.* also demonstrated previously that *Int* conjugated to the OVA H-$2K^b$ CTL epitope (SIINFEKL) is endocytosed via macropinocytosis by DCs in an ATP-dependent manner [14]. After internalization of Ag via macropinocytosis, Ag resides in macropinosomal vesicles. The macropinosomes move from the cell periphery, fuse with endosomes and mature lysosomes, or regurgitate the contents into the extracellular medium [30, 31]. Therefore, there are two possible mechanisms for the processing and cross-presentation of Ag delivered by R8-Lip. In the first mechanism, R8-Lip-OVA escapes from the macropinosomes or endosomes/lysosomes into the cytosolic compartment, where Ag accesses the conventional MHC class I pathway. Alternatively, R8-Lip-OVA are processed inside the hydrolytic compartments of endosomes/lysosomes, and the resulting peptide epitopes associate with pre-existing MHC class I molecules in the endosomes.

Earlier studies reported that CPPs, such as *Tat*, polyarginine and *Int,* escaped the endosomes and that release from the endosome was dependent on acidification of the endosomes [32, 33]. The endosomal compartment usually is acidic due to the action of

proton-pumping ATPase located in the endosomal membrane [34]. We found that an increase in endosomal pH, upon incubation with lysosomotropic agents (NH_4Cl and chloroquin), impaired the efficiency of cross-presentation (Fig. 5). These results suggested that the acidic environment of endosomes plays an important role in pMHC class I presentation of Ag encapsulating in R8-Lip. To elucidate the mechanism of Ag presentation in mDCs delivered by R8-Lip, the mature cells were incubated with lactacystin (a specific proteasome inhibitor) or leupeptin (an endosomal proteases inhibitor). Only lactacystin showed the markedly inhibition of pMHC presentation (Fig. 5), which indicates that Ag-processing of Ag delivered by R8-Lip would occur in the cytosol of mDCs rather than in endosomes/ lysosomes.

The difference in intracellular fate of R8-Lip-OVA between iDCs and mDCs likely affects differences in cross-presentation efficiency. It is possible that R8-Lip-OVA escaped from endosomes more efficiently in mDCs than in iDCs. Trombetta ***et al.*** reported that the induction of maturation lowers endosomal/ lysosomal pH by ~1 pH unit, from pH 5.5 to pH 4.5 [34, 35]. The acidified endosomes of mDCs may be preferable for R8-Lip-OVA escape from endosomal compartments. Consequently, Ag-processing and presentation are more efficient in transduced mDCs than in transduced iDCs.

In summary, the present study demonstrated that R8-Lip are an excellent delivery system for transducing DCs with protein or peptide Ag. The simplicity and versatility of R8-Lip preparation would be a potentially Ag transduction method into DCs, which the whole tumor associated Ag or even tumor lysates could be delivered instead of single minimal peptide epitopes. Notably, the transduction of DCs by R8-Lip

at the end of maturation process represents a logistical approach in the production of Ag-transduced DCs for clinical studies.

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Legends of Table and Figures

Table 1: Physical properties of Non-modified and Octaarginine (R8) modified liposomes encapsulating ovalbumin (OVA)

[a] Both type of liposomes were made of DOPE/CHEMS/EPC (7.5/1/1.5 molar ratio) and ovalubumin (OVA) was encapsulated into the liposomes. For R8-modified liposomes, the lipid membrane was modified with the cell penetrating peptide, R8 (7.5 mol%), to improve cellular uptake and intracellular trafficking.

[b] Polydispersity Index (P.D.) reflects the particle size distribution, ranging from 0.0 for entirely monodisperse particles, up to 1.0 for heterogenous particles.

Values are mean ± S.D. (n = 6).

Figure 1. Scheme for the transduction of DCs with octaarginine-modified liposomes encapsulating OVA (R8-Lip-OVA). (A) Transduction of iDCs, DCs were first pulsed with R8-Lip-OVA for 2 h, followed by induction of maturation by CpG-ODN treatment for 22 h. (B) Transduction of mDCs, DCs were matured for 22 h with CpG-ODN incubation followed by pulsing with R8-Lip-OVA for 2 h.

Figure 2. Level of cellular uptake of fluorescently-labeled R8-Lip or non-modified liposomes after transduction DCs. Cells were cultured in the presence of CpG-ODN (1 μg/ml) to induce maturation. At various time points, DCs were harvested and then pulsed with fluorescently-labeled liposomal formulations. Flow cytometry was performed. The mean fluorescence intensity (MFI-left axis) is indicated as the level of cellular uptake transduced by R8-Lip (white bar) or by non-modified liposomes (grey

bar). The immunophenotype analysis of DCs also was determined using CD86 expression on DCs, which is used as a maturation marker (right axis).

Figure 3. Presentation of OVA by MHC class I. (a) DCs, in either the immature or mature stage, were treated with 2 μg OVA in the form of solution, non-modified liposomes and R8-Lip. (b) Immature and mature DCs were incubated with various concentrations of R8-Lip-OVA, ranging from 0.025 to 0.1 mM final lipid concentration. For iDCs transduction (transduced 2 h → mat 22 h), the harvested DCs were transduced first with R8-Lip-OVA, then the cells were stimulated with CpG-ODN for 22 h. For mDCs transduction (mat 22 h → transduced 2 h), DCs were transduced with R8-Lip-OVA following differentiation to mature DCs. To determine MHC class I presentation, all transduced DCs were added to B3Z T cells for 15 h. *LacZ* activity in B3Z hybridoma cells was then assayed as described (see Materials and Methods). MHC class I presentation is expressed as a relative absorbance (595 nm) of DCs pretreated with OVA formulations compared to that of untreated DCs (without OVA loading). Data represent mean ± SD of at least three independent DCs cultures.

Figure 4. Induction of CTL in mice by DCs transduced with R8-Lip-OVA. (a) EG.7-specific CTL activity was induced in mice immunized with transduced DCs. A lower CTL response was detected in mice that were injected with DCs transduced with R8-Lip at immature stage (iDCs transduction) compared to that of mice receiving DCs transduced at mature stage (mDCs transduction). C57BL/6 mice (n = 4 in each group) were pre-immunized two times with iDCs or mDCs (7.5×10^5 cells) transduced with R8-Lip containing 5 μg OVA, according to the protocol shown in (b). Fourteen days after the first immunization, spleen cells from individual mice were re-stimulated *in*

vitro with mitomycin C-treated EG.7 cells and CTL reactivity with E.G.7 cells was determined using a Cr^{51} release assay. (■, mDCs transduction; □, iDCs transduction).

Figure 5. R8-Lip-OVA are endocytosed via macropinocytosis and acidic environment of endosomes is required for cytosolic release. DCs, either at immature or mature stage, were pre-incubated for 30 min with: amiloride (a specific macropinocytotic inhibitor, 0.5, 1 mM), lysosomotropic agents, i.e. NH_4Cl (50, 100 mM), and chloroquin (5, 20 μM), followed by incubation with R8-Lip-OVA and addition to B3Z T cells. *LacZ* activity in B3Z cells was assayed as indicated. Data are presented as percentage antigen presentation (MHC class I) from triplicate wells (± SD) by comparing presentation efficiency in the presence of inhibitors with that in the absence of inhibitors.

Figure 6. Antigen presentation after R8-Lip-OVA internalization reaches the conventional MHC class I presentation pathway. DCs at mature stage were preincubated for 30 min with lactacystin (1, 10, 25, 50 μM) or leupeptin (50, 100, 250, 500 μM). Pre-treated mDCs were then pulsed with R8-Lip-OVA in the presence of inhibitors for 2 h. The antigen presentation was then assayed by quantitative determination *LacZ* activity. Data are presented as percentage antigen presentation (MHC class I) from triplicate wells (± SD) by comparing presentation efficiency in the presence of inhibitors with that in the absence of inhibitors.

Figure 7. Confocal laser scanning microscopic image of immature (A) and mature DCs (B) after incubation with R8-Lip encapsulating FITC-labeled OVA (green fluorescent). The endosome/ lysosome compartments of DCs were stained with LysoTracker Red. The labeled-OVA co-localized with LysoTracker Red in immature DCs, as indicated by

yellow arrows. In contrast, in mDCs, the labeled-OVA did not co-localized with LysoTracker Red, as indicated by white arrows.