Modifying antigen-encapsulating liposomes with KALA facilitates MHC class-I antigen presentation and enhances anti-tumor effects

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ABSTRACT

For a successful anti-cancer vaccine, antigen presentation on the major histocompatibility complex (MHC) class-I is a requirement. To accomplish this, an antigen must be delivered to the cytoplasm by overcoming the endosome/lysosome is required. We previously reported that a lipid-nanoparticle modified with a KALA peptide (WEAKLAKALAKALAKHLAKALAKALKA), an α-helical cationic peptide, permits the encapsulated-pDNA to be efficiently delivered to the cytoplasm in bone marrow-derived dendritic cells (BMDCs). Herein, we report on the use of KALA-modified liposomes as an antigen carrier, in an attempt to induce potent antigen-specific cellular immunity. The subcutaneous injection of KALA-modified ovalbumin (OVA)-encapsulating liposomes (KALA-OVA-LPs) elicited a much more potent OVA-specific cytotoxic T-lymphocyte activity and anti-tumor effect in comparison with particles that were modified with octaarginine (R8), a cell-penetrating peptide (R8-OVA-LPs). In addition, the numbers of OVA-specific $CD8^+$ T cells were increased by immunization the KALA-OVA-LPs. The treatment of BMDCs with KALA-OVA-LPs induced a substantial MHC class-I antigen presentation. Furthermore, the

acidic pH-dependent membrane destabilization activity of KALA-OVA-LPs strongly suggests that they are able to escape from endosomes/lysosomes, and thereby deliver their cargos to the cytoplasm. Collectively, the KALA modified liposome is a potential antigen delivery platform for use as a protein vaccine.

INTRODUCTION

Antigen presentation on the major histocompatibility complex (MHC) class-I in antigen-presenting cells (APCs) (e.g. dendritic cells (DCs), macrophage) is particularly important for the induction of cellular immunity, an immunological action which plays a key role in effective cancer immunotherapy via the activation of antigen-specific cytotoxic T-lymphocytes (CTL).[1-5] An exogenous antigen is usually degraded into peptide fragments in endosome/lysosomes and presented in the context of MHC class-II, and thereby activate $CD4^+$ T cells for inducing humoral immunity. Therefore, a strategy for delivering the antigen to the cytoplasm is prerequisite for the effective activation of cellular immunity, since the antigen must be subjected to proteosomal degradation for antigen presentation onto the MHC class-I ("cross presentation").

To accomplish an efficient antigen delivery, considerable efforts have been devoted to develop various drug delivery carriers.[6-8] The liposome is one of the most useful carriers for antigens since they contain an inner aqueous phase in which water-soluble molecules can be

encapsulated.[9-11] Furthermore, when the surfaces of liposomes are modified with functional molecules (i.e. peptides, carbohydrates, and antibodies), this can facilitate the efficient delivery of an antigen.[6,10,12-15] For efficient cellular uptake, the use of cationic liposomes (i.e. N-[1-(2,3-Dioleoyloxy)propyl]-N, N, N-trimethylammonium methyl-sulfate: DOTAP) can be beneficial, since it can be inherently absorbed on the cellular surface. Moreover, it is well known that cationic nanoparticles are taken up by macrophages *in vitro* and *in vivo* (i.e. Kupffer cells).[16,17] We previously reported on the development of a liposome-based antigen carrier, octaarginine (R8)-modified liposomes.[18] Modification with R8, which mimics the TAT peptide of the influenza virus, enhanced endosomal escape, as well as the uptake of the liposomes in murine bone marrow-derived dendritic cells (BMDCs), compared with simple cationic liposomes. Furthermore, the R8-modified liposomes that contained encapsulated ovalbumin (OVA) elicited efficient antigen presentation in the context of MHC class-I and an OVA-specific anti-tumor effect, while conventional DOTAP liposomes preferred MHC class II-mediated antigen presentation. Thus, the rational design of functional molecules for delivering an antigen to the cytoplasm allowed us to develop an effective anti-tumor vaccine system.

We recently reported on the development of a KALA-modified lipid nanoparticle containing pDNA (a multi-functional envelope-type nano device: KALA-MEND) as a carrier of a DNA vaccine.[19,20] The key component (KALA) in this system adopts an α-helical structure at physiological pH for membrane destabilization.[21] To modify the liposomal structure with it, the peptide was stearylated. As a result, the KALA-MEND exhibited a >2 orders of magnitude higher gene transfection activity in comparison with R8-modified one (R8-MEND) in BMDC. Moreover, it unexpectedly induced the production of a large variety of cytokines and chemokines via the stimulation of cytosolic DNA sensors (i.e. TBK1/STING pathway, AIM-2 inflammasome). These results indicate that KALA-modified liposomes are more beneficial for delivering encapsulating cargos to the cytoplasm in comparison with R8-modified particles. This observation prompted us to evaluate the possibility of using the KALA peptide to deliver an antigen protein to cells for a cancer vaccine. The focus of this study was on the induction of the antigen presentation on MHC class-I molecules, and the resulting cellular immunity for cancer therapeutics.

RESULTS

Measurement of OVA-specific CTL activity in mice immunized with KALA-OVA-LPs

To evaluate the efficiency of the KALA peptide for antigen protein delivery, we designed KALA peptide-modified OVA-encapsulating liposomes (KALA-OVA-LPs). The liposomes were composed of 3 types of lipids; DOPE (a membrane fusogenic lipid for overcoming the biomembrane barrier), CHEMS (a pH-sensitive lipid for creating sensitivity to a low pH environment: endosomes/lysosomes and EPC (a membrane stabilizing lipid for the formation of liposomes), as reported previously.[18] To increase the membrane fusogenic activity of the liposomes, DOPE was employed in the lipid composition, while an EPC-based particle one was used as a non-fusogenic control, in order to clarify the relationship between membrane-fusogenic property and vaccine activity. The OVA protein was encapsulated in liposomes by repetitive freeze/thaw cycles. After liposomal formation, the surfaces of the liposomes were modified with the KALA peptide by adding a lipid derivative of the KALA peptide (STR-KALA). As a control for the cationic peptide, octaarginine (R8) was used to modify the particles, instead of KALA (R8-OVA-LPs). The physicochemical properties of each liposome preparation are shown in

Table 1. The modification with STR-KALA or STR-R8, resulted in a large increase in ζ-potential (approximately to +50 mV), while the size and polydispersity index (PdI) were comparable to the values for non-modified OVA-LPs (approximately 160-170 nm and 0.15, respectively).

In initial experiments, we evaluated the impact of the modification of the KALA peptide on the induction of antigen-specific cellular immunity *in vivo*. To investigate this, *in vivo* OVA-specific CTL activity was evaluated after subcutaneous immunization of the KALA-OVA-LPs. The liposomes containing the indicated amount of OVA, free-OVA or empty KALA-modified liposomes (KALA-LPs) were subcutaneously injected to the back of the necks of the mice 1 time. OVA-specific CTL activity was evaluated at 7 days after immunization (**Fig. 1**). First, immunization with the OVA protein *per se* did not induce CTL activity. In contrast, a dose-dependent increase in CTL activity was achieved when the OVA was immunized using the DOPE-incorporated KALA-OVA-LPs whereas the CTL activity for the R8-OVA-LPs was marginal, even in the case of a maximum dose (50 μg). Of note, the corresponding activities for non-modified OVA-LPs were at the background level. These data collectively indicate that KALA is a key component for the effective induction of CTL activity. While the EPC-incorporated KALA-OVA-LPs also induced significant CTL activity, the activity was

inferior to that for the DOPE-based KALA-OVA-LPs, suggesting that modification with the fusogenic lipid envelope and KALA synergistically potentiated the immune stimulative activity.[20] Furthermore, the KALA-OVA-LPs induced significant CTL activity by single immunization at a dose of 13 μg OVA, a smaller dose in comparison with previous reports.[22,23] Collectively, these data suggest that the DOPE-based KALA-OVA-LPs have the potential for use as an anti-cancer vaccine system.

Anti-tumor effect of KALA-OVA-LPs

To prove the potential of KALA-OVA-LPs as an anti-cancer vaccine, the prophylactic anti-tumor effect against E.G7-OVA, a mouse lymphoma EL4 expressing the OVA gene, was evaluated. Mice were immunized with KALA-OVA-LPs, R8-OVA-LPs, non-modified OVA-LPs (equivalent to 25 μg of OVA) or empty KALA-LPs (the same amount of lipid as KALA-OVA-LPs) subcutaneously 1 time. The immunized mice were inoculated with E.G7-OVA in the right flank 1 week after immunization. Tumor volume was then monitored for up to 27 days after tumor inoculation (**Fig. 2a**). The immunization by the non-modified OVA-LPs failed to inhibit tumor growth, suggesting that the induction of OVA-specific

immunity was insufficient. Consistent with data from a previous study, immunization with the R8-OVA-LPs inhibited tumor growth, although the effect was marginal at this dose. On the other hand, immunization with the KALA-OVA-LPs exhibited a significant anti-tumor effect: tumor growth was completely suppressed up to day 15, and furthermore, in 3 of 5 mice, tumor growth was not detectable, even on day 27 (**Fig. 2b**). Consequently, antigen-encapsulating KALA modified liposomes were more potent as an anti-cancer vaccine in comparison with the R8-modified particles.

To demonstrate the anti-tumor effect of the KALA-OVA-LPs in a more representative clinical setting, therapeutic anti-tumor experiments were carried out. Mice were inoculated with E.G7-OVA cells, followed by immunization with the KALA-OVA-LPs. Furthermore, to evaluate the synergistic effect of an immune checkpoint inhibitor on the therapeutic effect, mice were treated with an anti-PD-1 antibody when they were immunized with KALA-OVA-LPs. As a result, tumor volume tended to decrease on average as the result of immunization with the KALA-OVA-LPs (**Figure S1a**). Since the tumor volume of vehicle control animals was largely varied (tumor rejection occurred on day 20 in only 1 of 5 mice), no significant differences were observed. However, the administration of the KALA-OVA-LPs suppressed the tumor growth in four of five mice (under 500 mm^3 on day 20), whereas only two out of five mice were found to

be under 500 mm^3 on day 20 in the vehicle treatment group (**Figure S1b)**. Furthermore, while anti-PD-1 antibody-treated group reached an average of 1000 mm^3 on day 20, the group that had the combined KALA-OVA-LPs and anti-PD-1 treatment decreased on average by half (**Figure S1a**). These results suggest that KALA-OVA-LPs are potent in anti-tumor vaccines in a clinical setting and that they enhance the action of immune checkpoint inhibitors.

MHC class-I antigen presentation by KALA-OVA-LPs

To elucidate the mechanism responsible for the more prominent induction of *in vivo* OVA-specific immunity, we evaluated the antigen presentation activity of the KALA-OVA-LPs *in vitro.* In this analysis, BMDCs were used, since it is well known that DCs have a crucial role in the induction of antigen-specific immunity.[24-25] We first evaluated the innate immune activation of BMDCs in terms of enhancing the activation of CD80/86, markers of immune stimulation in BMDCs. However, no activation was observed even when KALA-OVA-LPs were administered (**Figure S2**). This is consistent with the fact that, an inflammatory immune response (IL-6 production in serum) was not observed after the injection of the KALA-OVA-LPs (**Figure S3)**. We then investigated a OVA-specific antigen presentation on MHC class-I. Antigen

presentation was quantified by co-cultivation with B3Z T-cell hybridoma, a reporter cell line that produces the lacZ protein in response to the $OVA_{257-264}$ epitope bound to H-$2K^b$.[26] As a result, modification with STR-KALA drastically enhanced antigen presentation from BMDCs, depending on the amount of modification (**Fig. 3a**). The effect of KALA modification approached saturation at 5mol% of total lipids, where almost no antigen presentation was observed in R8-OVA-LPs treatment at the corresponding density. Comparing the dose-response curves, the antigen presentation efficiency of the KALA-OVA-LPs at a dose of 16 μM approached those of R8-OVA-LPs at a dose of 128 μM, suggesting that KALA modification resulted in an enhancement in antigen presentation activity that was 8 times higher than that for R8 modification (**Fig. 3b**). The BMDCs treated with KALA-OVA-LPs elicited anti-tumor effects against E.G7-OVA when they were immunized via the footpad. This suggests that the enhanced antigen presentation in DCs is one of the mechanisms responsible for the anti-tumor activity of KALA-OVA-LPs (**Figure S4**). Furthermore, the enhancement of antigen presentation by modification with KALA was reduced when the lipid was replaced from DOPE to the non-fusogenic EPC (**Fig. 3a**). This result is consistent with the results of *in vivo* CTL assays. It appears that the KALA peptide is a potent activator for a liposomal antigen delivery system for the achievement of antigen presentation to MHC class-I.

***In vitro*/*in vivo* cellular uptake and membrane-fusogenic activity of the KALA-OVA-LPs**

To gain further insights into the mechanism underlying the difference in immunological action between KALA-OVA-LPs and R8-OVA-LPs, *in vitro*/*in vivo* cellular uptake and membrane-fusogenic activity were evaluated. The kinetics of the *ex vivo* cellular uptake of KALA-OVA-LPs and R8-OVA-LPs using BMDCs were first compared by means of flow cytometry. Liposomes encapsulating a fluorescence-labeled OVA (Alexa Fluor 488-conjugated OVA, 25% of whole OVA) were prepared to quantify the uptake of OVA. As shown in **Figure S5**, the uptake of both liposomes was observed on 6 and 24 hours after the treatment. Quantitative analysis revealed that the geomean values for the uptake of both liposomes were comparable after 5 hr, while the initial uptake speed (at 2 hours) was significantly higher in the case of the KALA-OVA-LPs (**Figure S5a**). The most significant difference was their homogeneity: the coefficient variance (CV) value for the cellular uptake of KALA-OVA-LPs was significantly lower compared with that of R8-OVA-LPs (**Figure S5b)**. Furthermore, the difference in CV values became larger with increasing time, dominantly due to the increase in

the corresponding value specifically in R8-OVA-LPs. This difference might be relevant with the anti-tumor effect of the immunization of *ex vivo* antigen-treated cells (**Figure S4**).

To elucidate the behavior of the liposomes *in vivo*, flow-cytometric analyses of draining lymph nodes were performed after an s.c. injection of the liposomes. As a result, both liposomes, which were labeled with a fluorescently labeled lipid, were found to have accumulated in the draining lymph nodes at comparable levels (**Figure S6a**). Furthermore, while the large fraction of the liposomes were taken up by $CD11c^{-}F4/80^{-}$ cells, which were expected to be B cells, a portion of the liposomes were taken up by $CD11c^{+}F4/80^{+}$ cells, which are likely dendritic cells or macrophages (**Figure S6b**). Importantly, the level of *in vivo* cellular uptake to these cells was comparable between the KALA-OVA-LPs and the R8-OVA-LPs. These results suggest that the uptake process was not a major determinant for explaining the difference in the *in vivo* antigen-specific immune response between the KALA-OVA-LPs and R8-OVA-LPs.

To investigate the membrane-fusogenic activity of the KALA-OVA-LPs and the R8-OVA-LPs under conditions of physiological or endosomal pH, the membrane disruption activity of RBCs was monitored after incubation with the liposomes by measuring the amount of hemoglobin that was released into the supernatant. In this experiments RBCs were mixed with various amounts of liposomes in PBS adjusted to pH 5.5, 6.5 and 7.4, which mimic late

endosomes, early/recycling endosomes, and the cytoplasm/extracellular space, respectively.[27] As a result, the KALA-OVA-LPs induced membrane disruption depending on the lipid concentration at pH 5.5 and 6.5, whereas membrane disruption was marginal in the case of the R8-OVA-LPs (**Fig. 4**). Furthermore, antigen presentation in the presence of chloroquine, an endosome disruptive agent, was evaluated, to investigate whether the membrane disruptive activity of the KALA peptide amplifies antigen presentation. As a result, the chloroquine treatment enhanced antigen presentation in cells that were treated with the R8-OVA-LPs, while the activity of the KALA-OVA-LPs was not enhanced, but rather decreased (**Fig. 5**). These results suggest that modifying liposomes with the KALA peptide induced a higher fusion with biomembranes in comparison with R8, especially under acidic pH conditions.

Immunological analysis of the mice immunized with the KALA-OVA-LPs

To clarify the precise mechanism responsible for the immune response induced by the KALA-OVA-LPs, we performed further immunological analyses *in vivo*. We first evaluated the contribution of CD8- and CD4-positive T cells to the final output of in vivo CTL activity by depleting these cells using an antibody (**Fig. 6a**). Mice were immunized with the KALA-OVA-LPs on day 0, and subsequently, 200 μg of an anti-CD4 or anti-CD8 antibody were

injected intraperitoneally on day –1, 1, 3, 6. OVA-specific CTL activity was evaluated one week after immunization by an *in vivo* CTL assay. Almost all of the CD4 or CD8 T cells were depleted on day 7. As a result, CD4 depletion failed to attenuate the CTL activity induced by the KALA-OVA-LPs, but rather slightly enhanced the effect. In contrast, CD8 depletion drastically impaired CTL activity. These collective results suggest that CD4 T cells were not involved in the antigen-specific CTL activity induced by the KALA-OVA-LPs. Thereafter, we also evaluated the number of OVA-specific T cells induced by immunization with the KALA-OVA-LPs and R8-OVA-LPs by staining for intracellular cytokines. To assess the frequency of OVA-specific $CD8^+$ T cells, splenocytes from immunized mice were isolated and stimulated by treatment with the $OVA_{257-264}$ peptide (MHC-I peptide) in the presence of a protein transport inhibitor, followed by staining for intracellular IFN-γ. As a result, immunization with the KALA-OVA-LPs significantly increased the frequency of IFN-γ^+CD8^+ T cells (**Fig. 6b**). This is consistent with the fact that the KALA-OVA-LPs induced significant in vivo CTL activity via $CD8^+$ T cells (**Fig.1, 6a**). Furthermore, stimulation of the $OVA_{323-339}$ peptide (MHC-II peptide) revealed that OVA-specific Th2 immunity was induced by immunization with KALA-OVA-LPs whereas Th1 and Th17 was not (**Fig. 6c**). Collectively, these results suggest that the numbers of OVA-specific

$CD8^+$ T cells and $CD4^+$ (Th2 phonotype) were expanded as the result of immunization with the KALA-OVA-LPs.

DISCUSSION

In the present study, we report on the development of a system that permits the efficient delivery of an antigen for cancer immunotherapy using an α-helical peptide: KALA. In general, positively charged nanoparticles are inherently taken up by a variety of cells, since they bind easily to the cell surface, which is negatively charged and covered by anionic proteoglycans.[28,29] Consistent with previous observations, both KALA-OVA-LPs and R8-OVA-LPs were efficiently taken up by BMDCs (**Figure S5a**). However, KALA-OVA-LPs were taken up more homogenously in comparison with R8-OVA-LPs, (**Figure S5b**). Since the surface charges and ζ-potentials of both particles (prepared with DOPE as a helper lipid) were quite comparable, these differences cannot be simply explained by the physicochemical characteristics. One possible reason is the differences in secondary structure, i,e, an α-helix structure. As reported previously, the KALA peptide forms an α-helical structure when the liposomal membrane exists, whereas the oligo-arginine peptide (especially in R8 and R9) forms a random coil structure.[21,30,31] Several previous studies have reported that the α-helical structure interacts with glycosaminoglycans, whose binding is independent of electrostatic interactions.[32,33] While these electrostatic and structural interactions might contribute to the efficient and homogeneous uptake

of the KALA-OVA-LPs in BMDCs, the extent of accumulation in the lymph node between the KALA-OVA-LPs and the R8-OVA-LPs was comparable *in vivo* (**Figure S6**). Thus, the homogenous uptake of the KALA-OVA-LPs might only contribute to applications of *ex vivo* protein vaccines (**Figure S4**).

The most drastic difference between the KALA-OVA-LPs and R8-OVA-LPs was their membrane-fusogenic activity, especially at acidic conditions (**Fig. 4**). It is plausible that this ability contributes to the remarkable induction of antigen presentation by KALA-OVA-LPs (**Fig. 3**) since this process is prerequisite for MHC class-I antigen presentation to release the antigen into the cytoplasm by overcoming endosome/lysosomes membranes. The experiments using chloroquine also strongly suggest that the KALA peptide amplifies endosomal escape followed by the higher cytosolic delivery of antigen protein to a higher degree in comparison with R8 (**Fig. 5**). However, the reason why the fusogenic ability of KALA was elicited at acidic conditions remains to be determined. Of note, the data reported herein are quite inconsistent with an original design and function of KALA peptide: it formed an α-helix at physiological pH. In fact, we also developed a KALA-MEND to allow the encapsulating pDNA to enter the nucleus via step-wise membrane fusion with the plasma membrane and the nuclear membrane at cytoplasmic pH (pH=7.4). Thus, the prominently higher gene transfection of the KALA-MEND was not derived

from the nuclear membrane fusion-mediated nuclear delivery of pDNA as we designed.[19] Wyman *et al*. reported that the KALA-peptide can form an α-helix structure even under conditions of acidic pH, when the peptide is present with liposomes whereas its structure changed to a random coil without liposomes.[21] Therefore, it is highly possible that the KALA might form a α-helix when it is modified onto the lipid envelope. Therefore, the pH-dependent membrane-fusogenic activity can be explained by assuming that the membrane-fusogenic activity might be perturbed via electrostatic interactions between KALA and the negatively charged helper lipid (CHEMS) that is incorporated in the liposomal component. However, when the KALA-LPs were exposed in an acidic pH in a range close to its pKa (~5.8), the fraction of ionized CHEMS is decreased by the protonation of the carboxyl group. In this situation, the electrostatic interactions become less intense, and the fusogenic function of KALA would be recovered. Such a pH-dependent loss of negative charge was conventionally used as a trigger for endosomal membrane fusion via the formation of an inverted hexagonal H_{II} phase in liposomes composed of DOPE, a cone-shape lipid and CHEMS.[34,35] Collectively, the KALA-OVA-LPs might achieve an accelerated endosomal escape via a dual mechanism in response to pH via the protonation of CHEMS: switching on the KALA function by cancelling the electrostatic interactions with the peptide, and the loss of lamellar structure to form an inverse cone packaging

formation for membrane fusion. Another possible reason is the presence of a histidine (His) residue in KALA. It is known that a His residue can be employed to facilitate viral membrane fusion in response to a low pH in some α-helical viral proteins.[36,37] Further investigations will be needed in order to clarify the effect of His residues on the membrane fusogenic property of the KALA peptide.

As shown in **Figure 1** and **3a**, the induction of CTL activity and antigen presentation was weak in EPC-based liposomes, presumably because EPC-based liposome lacks membrane fusiogenic activity. Phosphatidylcholine derivatives, including EPC are considered to be a stabilizer of lipid bilayers, since they form a cylindrical structure in the lipid membrane. Thus, it is difficult to overcome the endosomal membrane barrier, and subsequently for the antigen to be released to the cytoplasm. To support this. we previously demonstrated that KALA-modified DOPE liposomes that encapsulate pDNA could stimulate the BMDCs via the stimulation of cytosolic DNA sensors, whereas the EPC-based showed no activity.[20] Thus, EPC is inadequate in terms of cytoplasmic delivery, at least when used in combination with KALA peptides.

As shown in **Figure 3**, KALA-OVA-LPs induced potent antigen presentation in BMDCs. In addition, subsequent immunization with these DCs (*ex vivo* immunization) efficiently inhibited tumor growth in comparison with those of R8-OVA-LPs treated BMDCs (**Figure S4**). However,

this *ex vivo* approach conferred less anti-tumor effect in comparison with a direct *in vivo* immunization of KALA-OVA-LPs: the complete prevention of the tumor growth was observed up to day 15 after tumor inoculation (**Fig. 2a**). These data suggest that DCs are not a unique cell population that contributes to the anti-tumor effect of the KALA-OVA-LPs *in vivo*. One of the considerable populations is macrophages, a type of APC as well as DCs. In fact, while the large fraction of the liposomes was taken up by $CD11c^{-}F4/80^{-}$ cells, which have the characteristics of B cells, a part of the liposomes was taken up by $CD11c^{+}F4/80^{+}$ cells, which is considered to be dendritic cells/macrophages (**Figure S6b**). It is generally thought that the DCs are major, even unique, APCs that can activate naïve T cells, and macrophages are considered to be less potent APCs in terms of their activation. However, an accumulating body of evidence has revealed that a specific subpopulation of macrophages in lymph nodes, CD169-positive macrophage, contribute to the antigen-specific CTL response and subsequent anti-tumor effect.[38-40] For example, Muraoka *et al.* revealed that an antigen peptide encapsulated within a nanoparticulate hydrogel (nanogel, composed of cholesteryl pullulan) was efficiently incorporated into CD169-positive medullary macrophages and elicited a potent anti-tumor effect.[40] Although a cationic nanogel was reported to be less incorporated into macrophages in draining lymph nodes in comparison with neutral ones, it has also been reported that a cationic agarose hydrogel could

deliver incorporated-nucleic acids into CD169-positive macrophages.[41] These facts suggest that macrophages, in particular CD169-positive macrophages, may contribute to the anti-tumor effect of KALA-OVA-LPs *in vivo*. Additional strategies for targeting draining lymph nodes may improve the efficiency of the KALA-OVA-LPs, such as PEGylation for shielding the surface charge to avoid trapping by the extracellular matrix or ligand modification for the targeting of the specific cell type.[42,43]

In conclusion, we report herein on the development of a carrier for delivering an antigen protein that has the potential for use in cancer immunotherapy. Modifying antigen-encapsulating liposomes with KALA facilitated antigen-specific CTL activity and anti-tumor effects when the liposomes were administered subcutaneously. Furthermore, OVA-specific $CD8^+$ T cells and $CD4^+$ cells (Th2 phenotype) were expanded by immunization with the KALA-OVA-LPs. Considering that $CD4^+$ T cells were not involved in OVA-specific CTL activity induced by the KALA-OVA-LPs (**Fig. 6a**), the activation of Th2 immunity might not relevant with the activity of the KALA-OVA-LPs as anti-cancer vaccine. However, this result suggests that a possibility for an application of the KALA-OVA-LPs to infectious diseases via induction of humoral immunity. Experiments using BMDCs suggested that the KALA-OVA-LPs induced a much

more potent OVA-specific MHC class-I restricted antigen presentation in comparison with R8-OVA-LPs, that were reported to be a highly efficient carrier. This effect can be attributed to the cytoplasmic delivery of the antigen via the acidic environment-specific membrane destabilization activity in the KALA-OVA-LPs. Collectively, the KALA-modified antigen-encapsulating liposomes are promising devices for use as an anti-cancer vaccine, which can be combined with immune checkpoint inhibitors such as an anti-PD-1 antibody.

MATERIALS AND METHODS

Materials

1,2-dioleoyl sn-glycero-3-phosphoethanolamine (DOPE) and egg phosphatidylcholine (EPC) were purchased from Avanti Polar Lipid (Alabaster, AL, USA). Cholesterol (Chol), cholesteryl hemisuccinate (CHEMS), ovalbumin (OVA, grade VI) were purchased from Sigma (St. Louis, MO, USA). Alexa Fluor® 488 conjugated OVA and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine,4-chlorobenzenesulfonate salt (DiD) were purchased from Invitrogen (Carlsbad, CA, USA). Chlorophenol red β-D-galactopyranoside was purchased from Roche Diagnostics (Minneapolis, MN, USA). Stearylated octaarginine (STR-R8) and stearylated KALA (STR-KALA) were custom-synthesized by Kurabo (Osaka, Japan) as described previously.[21] Mouse recombinant granulocyte-macrophage stimulating factor (GM-CSF) and Mouse IL-6 Quantikine ELISA were purchased from R&D systems (Minneapolis, MN, USA). Fluorescent dye-conjugated monoclonal antibodies, including F4/80 (Clone: BM8, Cat#: 123109), CD11c (Clone: N418, Cat#: 117305), CD3 (Clone: 145-2C11, Cat#: 100311), CD4 (Clone: RM4-5, Cat#: 100511), CD8 (Clone: 53-6.7, Cat#: 100705), IFNγ (Clone: XMG1.2,

Cat#: 505807), IL-4 (Clone: 11B11, Cat#: 504103), IL-17 (Clone: TC11-18H10.1, Cat#: 506903), CD80 (Clone: 16-10A1, Cat#: 104707), CD86 (Clone: GL-1, Cat#: 105007), isotype control antibody and Anti-mouse CD16/32 antibody (Clone: 93. Cat#: 101302) were purchased from Biolegend (San Diego, CA, USA). OVA H-2K^{b} cytotoxic T-lymphocyte epitope peptide (SIINFEKL, $OVA_{257-264}$) was synthesized by TORAY research center, Inc. (Tokyo, Japan). OVA H-2K^{b} helper T-lymphocyte epitope peptide (ISQAVHAAHAEINEAGR, $OVA_{323-339}$) was synthesized by Invitrogen. All other chemicals were commercially available and reagent grade products.

Cell lines

E.G7-OVA cells, a murine lymphoma cell line EL4 expressing chicken OVA, were purchased from the American Type Culture Collection (Manassas, VA, USA).B3Z, a CD8^{+} T-cell hybridoma specific for the $OVA_{257-264}$ epitope in the context of K$^{b,41\ 26}$, were a generous gift from Dr. Shastri (University of California, Berkeley, CA, USA). These cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium containing 50 μM 2-mercaptethanol, 10 mM

HEPES, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal calf serum.

Preparation of KALA-OVA-LPs and R8-OVA-LPs

DOPE-based OVA-LPs were composed of DOPE, CHEMS, EPC (7.5:1.25:23.75 molar ratio) and EPC based OVA-LPs were composed of EPC, CHEMS, Chol (7:1:2). Each of the OVA-LPs were prepared by the lipid film hydration method as reported previously with minor modifications.[18] Briefly, a chloroform solution of lipids was mixed in a test tube, and the solvent was evaporated by a stream on nitrogen gas to produce a thin lipid film. The resulting lipid film was hydrated with a 5 mg/mL solution of OVA in 10 mM HEPES (pH 7.4) for 10 minutes at room temperature (300 μL, 10 mM of total lipid concentration). For FACS analysis, 25% of the OVA was substituted by an Alexa Fluor® 488 conjugated one. The hydrated lipid film was then gently sonicated to produce the liposomes. The liposome suspension was subjected to five freeze/thaw cycles. After the treatment, the liposome suspension was extruded through polycarbonate membrane filters (400 nm pore size; Nucleopore) with a Mini-extruder (Avanti Polar Lipids) for sizing the liposomes. To remove unencapsulated OVA, the liposome

suspension was centrifuged at 80,000 *g* for 30 minutes at 4 degrees. After purification of the liposomes, the surface of the liposomal membrane was modified with STR-KALA and STR-R8 (5 mol% of total lipids) by vortexing the liposome suspension. The diameter and ζ-potential of the liposomes were determined using an electrophoretic light-scattering spectrophotometer (Zetasizer; Malvern Instruments Ltd., Malvern, WR, UK). The concentration of lipid and protein were determined using a phospholipid assay kit (Wako, Osaka, Japan) and a BCA protein assay kit (PIERCE, Rockford, IL, USA) after precipitation.[44]

Tumor challenge

Female C57BL/6J (H-2^b) mice (6-8 weeks old) were obtained from Japan SLC (Shizuoka, Japan). The protocol for using the mice was approved by the Pharmaceutical Science Animal Committee of Hokkaido University. In the prophylactic experiment, the mice were immunized with KALA-OVA-LPs, R8-OVA-LPs, unmodified OVA-LPs containing 25 μg OVA and KALA-LPs (the same amount of lipid as KALA-OVA-LPs) subcutaneously. At 7 days after immunization, the left flank of the mice were subcutaneously inoculated with 8.0×10^5 E.G7-OVA cells. In the therapeutic experiment, the mice were subcutaneously inoculated on the

left flank with 8.0×10^5 E.G7-OVA cells. At 5, 9, 13 and 17 days after tumor inoculation, the mice were subcutaneously immunized with the KALA-OVA-LPs containing 25 μg OVA. Anti-PD-1 or isotype control antibody (Clone: RMP1-14, 2A3, Cat#: BE0146, BE0089, respectively, BioXCell (West Lebanon, NH, USA)) also administered intraperitoneally at a dose of 50 μg at 2 day intervals after the immunization. Tumor volume was calculated by the following formula: major axis × minor axis^2 × 0.52

***In vivo* CTL assay**

In vivo CTL assays were performed as described previously.[45] Briefly, C57BL/6J mice were subcutaneously immunized with each sample. In the CD4/CD8 depletion experiments, 200 μg of anti-CD4, CD8 or isotype control antibody (Clone: YTS 191, YTS 169.4 and LTF-2, Cat#: BE0119, BE0117, and BE0090, respectively, BioXcell) was administered intraperitoneally at 1 day before and 1, 3, 6 days after immunization. After 7 days, splenocytes were prepared from naive C57BL/6J mice and incubated for 1 hour at 37 degrees with the $OVA_{257\text{-}264}$ peptide in RPMI-1640 medium containing 50 μM 2-mercaptethanol, 10 mM HEPES, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal calf serum. The

$OVA_{257-264}$ peptide-presented splenocytes were then labeled by incubation for 10 minutes at 37 degrees with 5 μM carboxyfluorescein succinimidyl ester (CFSE) in PBS ($CFSE^{High}$ cells). The naïve splenocytes were labeled by incubation for 10 minutes at 37 degrees with 0.5μM CFSE in PBS ($CFSE^{Low}$ cells). CFSE-labeled cells were washed with PBS. A mixture of 5×10^6 cells $CFSE^{High}$ cells and 5×10^6 cells $CFSE^{Low}$ cells was intravenously injected into the immunized mice. After 20 hours, splenocytes from immunized mice were collected, and single-cell suspensions were analyzed for the detection and quantification of CFSE-labeled cells by FACSCaliber™ (BD, Franklin Lakes, NJ, USA). The numbers of $CFSE^{Low}$ cells were essentially the same in all samples including the non-treated group. The values for lysis were the number of $CFSE^{High}$ cells corrected by the number of $CFSE^{Low}$ cells. In this study, no non-specific lysis was observed.

Measurement of IL-6 concentration in serum

The KALA-OVA-LPs and the R8-OVA-LPs were subcutaneously injected to the backs of C57BL/6J mice. After, 1, 3, 6, 12 and 24 hours, blood was collected from the tail vein, followed

by centrifugation at 2,000 g, for 10 minutes at 4 degrees to obtain serum samples. IL-6 concentration of the serum was measured by a Mouse IL-6 Quantikine ELISA Kit.

Lymph nodes accumulation of the liposomes

To evaluate the lymph node accumulation of each of the OVA-LPs, 0.1 mol% DiD-labeled each OVA-LPs were subcutaneously injected to the left and right flank of C57BL6/J mice. After 24 hours, inguinal lymph nodes were collected, mashed, and filtered through a nylon mesh. The acquired cell suspension was washed with FACS buffer twice, followed by Fc-blocking (anti-CD16/32 antibody) and staining with PE-conjugated anti-F4/80 (2.5 μg/mL) and FITC-conjugated CD11c (5.0 μg/mL) antibody. After washing, the cells were analyzed by FACSAriaII (Becton Dickinson). Dead cells were removed by means of a 7-AAD Viability Staining Solution (Biolegend).

Preparation of mouse BMDCs

BMDCs of mice were prepared as described previously.[18] Briefly, bone marrow cells were cultured overnight in RPMI1640 medium containing 50 μM 2-mercaptethanol, 10 mM HEEPS, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal calf serum. Non-adherent cells were harvested and cultured in the same medium supplemented with 10 ng/mL GM-CSF. On days 2 and 4, non-adherent cells were removed, and the remaining adherent cells were cultured in fresh medium containing 10 ng/mL GM-CSF. On days 6, non-adherent cells were used in experiment as immature BMDC.

Antigen presentation assay

BMDCs (1.0×10^6 cells) were incubated with each OVA-LPs at an indicated lipid concentration for 2 hours at 37 degrees in serum-free RPMI-1640 medium. RPMI-1640 medium was then added to the cells, followed by a further 3 hours of incubation. GM-CSF was added in the medium at 10 ng/mL throughout the incubation. In the chloroquine experiments, the BMDCs were incubated with medium containing chloroquine for 30 minutes before being incubated with the liposomes. The treated BMDCs were harvested by pipetting, followed by centrifugation at 500 *g*, for 5 minutes. The BMDCs (2.0×10^5 cells) were mixed with B3Z (1.0×10^5 cells) and

co-cultured in RPMI-1640 with 10% FCS in 96 well plate at 37 degrees for 15 hours. The co-cultured cells were washed with PBS, followed by incubation with 100 μL chlorophenol red β-D-garactopyranoside buffer (5 mM chlorophenol red β-D-garactopyranoside, 0.125% NP-40 and 9 mM $MgCl_2$ in PBS) for 4 hours at 37 degrees. After the incubation, the absorbance at 595 nm of each well was measured by a microplate reader (Benchmark Plus; BioRad, Hercules, CA, USA).

Flow cytometric analysis of BMDCs

To evaluate the kinetics of uptake of each OVA-LPs, BMDCs were incubated with the OVA-LPs encapsulated Alexa Fluor® 488 conjugated OVA in serum-free RPMI-1640 for 2 hours, followed by a further 0, 4, 10, 22 hours (total incubation time was 2, 6, 12, 24 hours, respectively). GM-CSF was added in the medium at 10 ng/mL throughout the incubation. After the incubation, the cells were harvested by pipetting, followed by washing with RPMI-1640 medium 2 times, and PBS containing 0.5% bovine serum albumin and 0.1% NaN_3 (FACS buffer) 2 times. After washing, the BMDCs were analyzed by FACSCalibur™ (Becton Dickinson, Franklin Lakes, NJ, USA).

Hemolysis assay

Hemolysis assays were performed as described previously with minor modifications.[46] Briefly, male ICR mice (4-6 weeks) were obtained from Japan SLC. Fresh red blood cells (RBCs) were collected from ICR mice and suspended in PBS (pH 7.4) or 10 mM malic acid/PBS (pH 5.5 or 6.5). The RBC suspension was mixed with indicated concentration of OVA-LPs, and then incubated at 37 degrees for 30 minutes. After the incubation, the absorbance of the supernatant at 545 nm was measured after centrifugation at 500 *g*, for 5 minutes. The RBCs lysed by incubation with 0.02 %(w/v) Triton-X was used as a positive control. As a negative control, the RBCs without OVA-LPs were also measured. The % lysis was represented as the % of the absorbance of positive control.

Intracellular cytokine staining assay

C57BL/6J mice were immunized with the KALA-OVA-LPs or the R8-OVA-LPs at a dose of 25 μg OVA twice every 7 days. At 7 days after the second immunization, splenocytes were harvested and incubated with 1 mM $OVA_{257-264}$ (for MHC class-I stimulation) or $OVA_{323-339}$ (for

MHC class-II stimulation for 1 hour at 37 degrees, followed by an additional 6 hours of incubation with GoldiPlug (BD). Cells were treated with anti-mouse CD16/32 antibody to block non-specific antibody binding, followed by staining the surface markers with APC-conjugated anti-CD3 (2.5 μg/mL) and FITC-conjugated anti-CD4 or CD8 (2.5 μg/mL) antibody in FACS buffer for 30 minutes at 4 degrees. Permeabilization/fixation was then performed using a Transcription Factor Buffer Set (BD) according to the manufacturer's protocol. Briefly, the stained cells were incubated in the Fix/Perm Working Solution for 45 minutes at 4 degrees. The cells were then washed twice with the Perm/Wash Working Solution, followed by staining the intracellular cytokines by PE-conjugated anti-IFNγ, IL-4, or IL-17 (1.2 μg/mL) antibody in Perm/Wash Working Solution for 45 minutes at 4 degrees, and analyzed by FACSAriaII (BD).

Statistical analysis

Comparisons between multiple treatment were performed by one-way analysis of variance, followed by Bonferroni correction. Comparisons between two treatments were performed by unpaired *t*-test. P value of <0.05 was considered to be a significant difference.

AUTHER CONTRIBUTION

N.M. and N.T. performed the experiments. N.M. and H.A. analyzed data and wrote the manuscript with critical input from the coauthors. H.A., T.N. and H.H. supervised the project and designed experiments. All authors contributed to discussing the results. All authors have given approval to the final version of the manuscript.

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Conflict of interest statement. None declared.

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FIGURE LEGENDS

Figure 1. *In vivo* CTL assay. C57BL/6 mice were immunized subcutaneously once with KALA-OVA-LPs, R8-OVA-LPs, OVA-LPs (DOPE or EPC as a helper lipid) or free OVA protein at a dose of 3.1, 13, 25 (KALA-OVA-LPs only) or 50 μg OVA. Fluorescent-labeled target cells ($OVA_{257-264}$ pulsed, $CFSE^{High}$) and control cells (no peptide pulsed, $CFSE^{Low}$) were injected intravenously at one week after immunization. The OVA-specific lysis was calculated from target cell / control cell ratio measured by flow cytometer 20 hours after injection. Data are mean + SEM (n = 3-5).

Figure 2. Anti-tumor effect of OVA-encapsulating liposomes. (a) C57BL/6 mice were immunized subcutaneously once with KALA-OVA-LPs, R8-OVA-LPs, OVA-LPs or KALA-LPs at a dose of 25 μg OVA. For the injection of KALA-LPs (empty liposomes), the dose of the lipid was adjusted to that for the the KALA-OVA-LPs. One week after immunization, mice were inoculated with 8.0×10^5 cells of E.G7-OVA in the left flank. The tumor volume was measured up to 27 days after inoculation. The plots represent the mean ± SEM (n = 5-6).

Statistical analyses were performed by the one-way ANOVA, followed by Bonferroni test. *P < 0.05 versus PBS group. **(b)** The tumor volumes in individual mice on day 27 were plotted.

Figure 3. Evaluation of MHC class-I antigen presentation. (a) BMDCs were treated with several types of KALA-OVA-LPs (DOPE or EPC as a helper lipid), modified with the KALA peptide (1, 3, 5, 7, 9 mol% of total lipid) or R8-OVA-LPs (DOPE as a helper lipid, modified with R8 peptide (5 mol% of total lipid) at a dose of 10 μM of lipid. After 5 hours, the treated cells were co-cultured with a B3Z T-cell hybridoma, specific for the $OVA_{257-264}$ epitope in the context of $K^{b,41}$ for 15 hours at 37 degrees. The co-cultured cells were lysed and incubated with chrolophenol red β-D-galactopyranoside buffer for 4 hours at 37 degrees. The absorbance at 595 nm was used as an index for antigen presentation activity. Data are mean + SD (n = 3). Statistical analyses were performed by the one-way ANOVA, followed by Dunnett test. **P < 0.01 versus 5 mol% KALA-OVA-LPs composed of DOPE. N.S.: not significant. **(b)** BMDCs were treated with the KALA-OVA-LPs or R8-OVA-LPs at several doses. Data are mean ± SD (n = 3) Statistical analyses were performed by Student's *t*-test. **P < 0.01.

Figure 4. **pH-dependent membrane destabilization activity of the KALA-OVA-LPs and the R8-OVA-LPs**. RBCs were incubated with the KALA-OVA-LPs and the R8-OVA-LPs at various pH values for 30 minutes at 37 degrees. The values are represented as relative values of the positive control acquired by Triton X-100 treatment. Data are mean + SD (n=3).

Figure 5. Antigen presentation activity in the presence of chloroquine; an endosome disruptive agent. BMDCs were pre-incubated with 0, 25, 50 μM chloroquine in medium, followed by treatment of the KALA-OVA-LPs or the R8-OVA-LPs at a lipid dose of 32 μM. After 5 hours, the treated cells were co-cultured with a B3Z T-cell hybridoma for 15 hours at 37 degrees. The co-cultured cells were lysed and incubated with chrolophenol red β-D-galactopyranoside buffer for 4 hours at 37 degrees. The absorbance at 595 nm was used as an index for antigen presentation activity. Data are the mean + SD (n = 6).

Figure 6. **Immunological analysis of the mice immunized with the KALA-OVA-LPs. (a)** C57BL/6J mice were immunized subcutaneously once with KALA-OVA-LPs at a dose of 25 μg OVA. At 1 days before and 1, 3, 6 days after the immunization, anti-CD4, anti-CD8 or isotype

control antibody was intraperitoneally administered at a dose of 200 μg. Fluorescent-labeled target cells ($OVA_{257-264}$ pulsed, $CFSE^{High}$) and control cells (no peptide pulsed, $CFSE^{Low}$) were injected intravenously at one week after immunization. The OVA-specific lysis was calculated from target cell / control cell ratio measured by flow cytometer 20 hours after injection. Data are mean + SEM (n = 3). Statistical analysis was performed by one-way ANOVA, followed by Bonferroni test. $^{**}P < 0.01$ versus Isotype control group. N.S.: not significant. **(b, c)** C57BL/6J mice were immunized subcutaneously with KALA-OVA-LPs at a dose of 25 μg OVA twice every 7 days. At 7 days after the second immunization, splenocytes were harvested and re-stimulated by (b) $OVA_{257-264}$ or (c) $OVA_{323-339}$, followed by an additional incubation with the protein transport inhibitor. Populations of cells in the spleen, which were positive in IFNγ (b, c), IL-4, and IL-17 (c) were quantified by flow cytometry after the immune-staining for intracellular cytokines. Each dot represents the percent of cytokine-positive cells in an individual mouse. Statistical analysis was performed by one-way ANOVA, followed by Bonferroni test. $^{*}P < 0.05$, $^{**}P < 0.01$ versus Non-treat group.

Supplemental Figure 1. Therapeutic anti-tumor effect combined with anti-PD-1 antibody treatment. C57BL/6 mice were inoculated in the left flank with 8.0×10^5 cells of E.G7-OVA. At 5, 9, 13, 17 days after inoculation, the mice were immunized subcutaneously with KALA-OVA-LPs at a dose of 25 μg OVA. An anti-PD-1 or a Isotype Ctrl antibody was also administered intraperitoneally at a dose of 50 μg at two day intervals after the first immunization. Tumor volume was measured up to 20 days after inoculation. The plots represent the mean ± SEM (n = 5). Tumor volume of an individual mouse of each group in the experiment.

Supplemental Figure 2. CD80/86 expression in BMDCs that were treated with liposomes. BMDCs (1.0×10^6 cells) were treated with the KALA-OVA-LPs, the R8-OVA-LPs or the non-modified OVA-LPs at a lipid dose of 32 μM. After 18 hours, the BMDCs were recovered and stained by PE-labeled anti-mouse CD80 and CD86 (Biolegend).

Supplemental Figure 3. IL-6 concentration in serum after liposome injection. C57BL/6 mice were subcutaneously administered KALA-OVA-LPs, R8-OVA-LPs at a dose of 25 μg OVA.

Blood from these mice was collected at 1, 3, 6, 12 and 24 hours after administration. Serum was separated from blood and the IL-6 concentration was measured by ELISA.

Supplemental Figure 4. ***Ex vivo*** **anti-tumor experiment.** BMDCs (1.0×10^6 cells) were treated with the KALA-OVA-LPs or R8-OVA-LPs at a lipid dose of 32 μM. After 6 hr incubation, the BMDCs were harvested. C57BL/6 mice were immunized with 5.0×10^5 cells of the harvested BMDCs treated with the KALA-OVA-LPs or the R8-OVA-LPs or non-treated BMDCs. At one week after immunization, mice were inoculated with 8.0×10^5 cells of E.G7-OVA in the left flank. Tumor volume was measured up to 23 days after inoculation. The plots represent the mean ± SEM (n = 4-5).

Supplemental figure 5. Uptake of KALA-OVA-LPs and R8-OVA-LPs in BMDCs. BMDCs were treated with KALA-OVA-LPs or R8-OVA-LPs encapsulating Alexa Flour 488-labeled OVA (25% of total OVA) at a lipid dose of 32 μM. After 2, 6, 12 or 24 hours, the BMDCs were recovered and measured the fluorescent intensity by flowcytometer. (a) Typical histogram of BMDCs treated with KALA-OVA-LPs or R8-OVA-LPs. (b) Average of fluorescence intensity

(Geo mean, left) and coefficient variance (CV) value (right). Data are mean ± SD (n=3). Statistical analyses were performed by Student's t-test. **P < 0.01.

Supplemental figure 6. Lymph node accumulation of the KALA-OVA-LPs and the R8-OVA-LPs. C57BL/6 mice were subcutaneously administered KALA-OVA-LPs, R8-OVA-LP modified with 0.1 mol% DiD at a dose of 25 μg OVA in the both flanks. After 24 hours, draining lymph nodes (inguinal lymph nodes) were isolated and homogenized. A nylon mesh-filtered cell suspension was analyzed by flow cytometry for the uptake of the fluorescently-labeled liposomes and the expression of F4/80 and CD11c. Data are the mean + SD (n=3). Statistical analyses were performed by the Student's t-test. N.S.: Not significant.

Liposome	Size (nm)	ζ-potential (mV)
OVA-Lp (DOPE)	**158 ± 2**	**−19 ± 1**
OVA-Lp (EPC)	**162 ± 1**	**−37 ± 11**
KALA-OVA-Lp (DOPE)	**169 ± 4**	**50 ± 1**
KALA-OVA-Lp (EPC)	**185 ± 32**	**30 ± 1**
R8-OVA-Lp (DOPE)	**178 ± 4**	**52 ± 1**
R8-OVA-Lp (EPC)	**169 ± 2**	**52 ± 4**

Table 1. Physicochemical properties of the various OVA-LPs.

Data were represented as the mean ± SEM value of at least three independent experiments (n=3-15)

Figure 1.

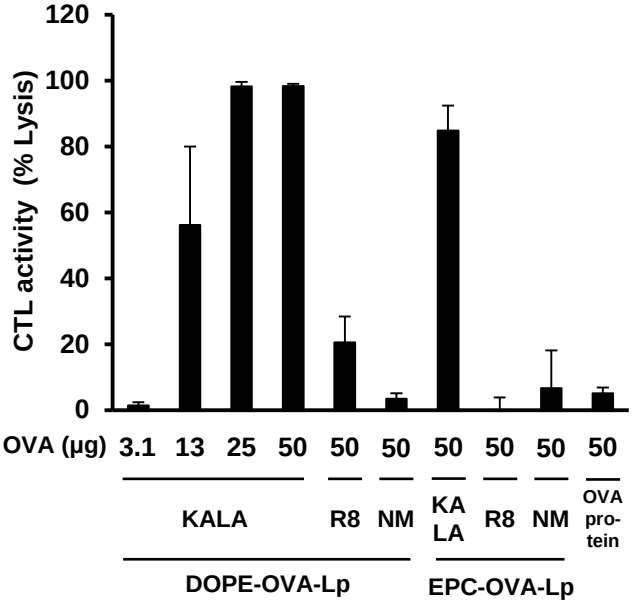

120
100
80
60
40
20
0
CTL activity (% Lysis)
OVA (μg)
3.1
13
25
50
50
50
50
50
50
50
KALA
R8
NM
KA LA
R8
NM
OVA pro-tein
DOPE-OVA-Lp
EPC-OVA-Lp

Figure 2.

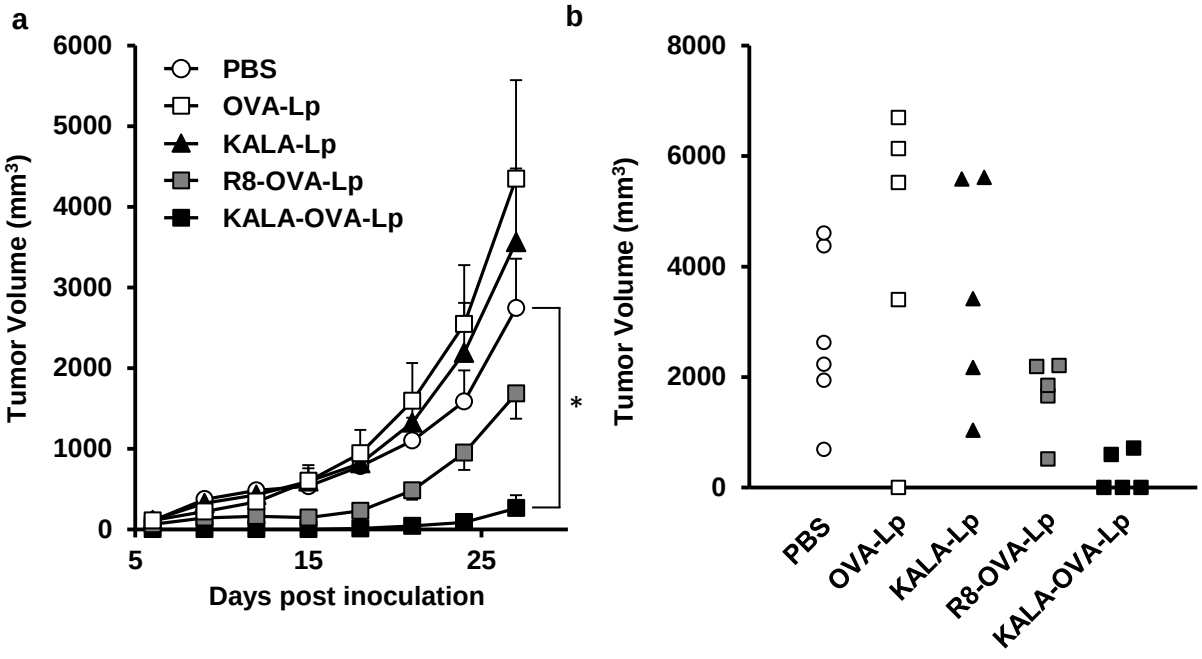

a
b
PBS
OVA-Lp
KALA-Lp
R8-OVA-Lp
KALA-OVA-Lp
Tumor Volume (mm3)
Days post inoculation
*
0
1000
2000
3000
4000
5000
6000
8000
5
15
25

Figure 3.

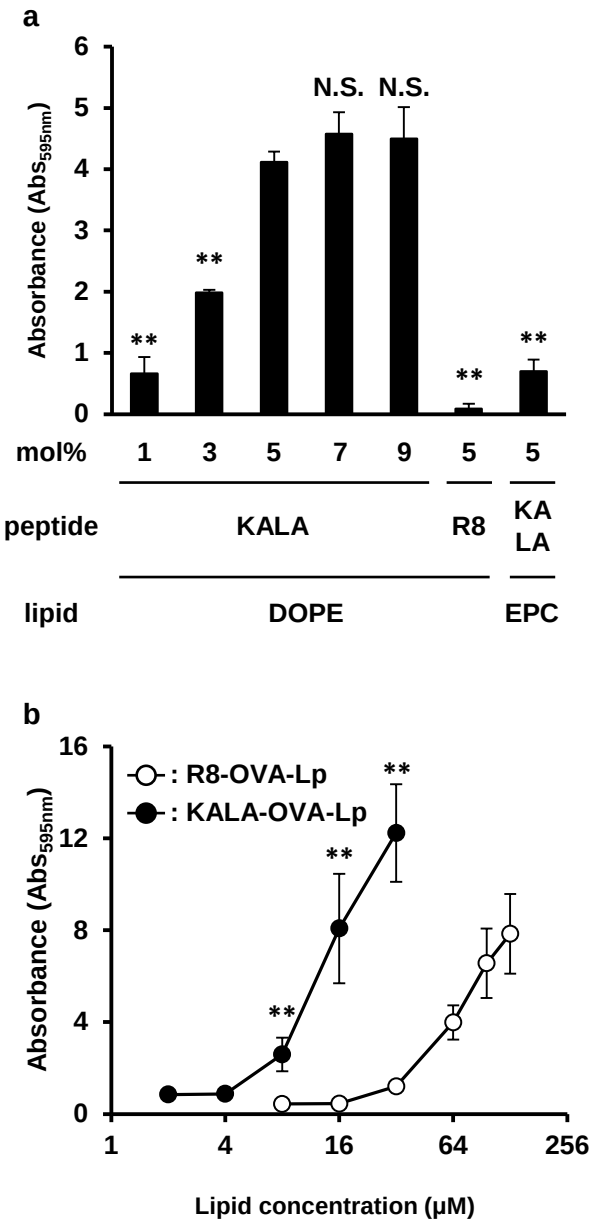

a
Absorbance (Abs595nm)
6
5
4
3
2
1
0
N.S.
N.S.
**
**
**
**
**
mol%
1
3
5
7
9
5
5
peptide
KALA
R8
KA
LA
lipid
DOPE
EPC
b
Absorbance (Abs595nm)
16
12
8
4
0
: R8-OVA-Lp
: KALA-OVA-Lp
**
**
**
1
4
16
64
256
Lipid concentration (μM)

Figure 4.

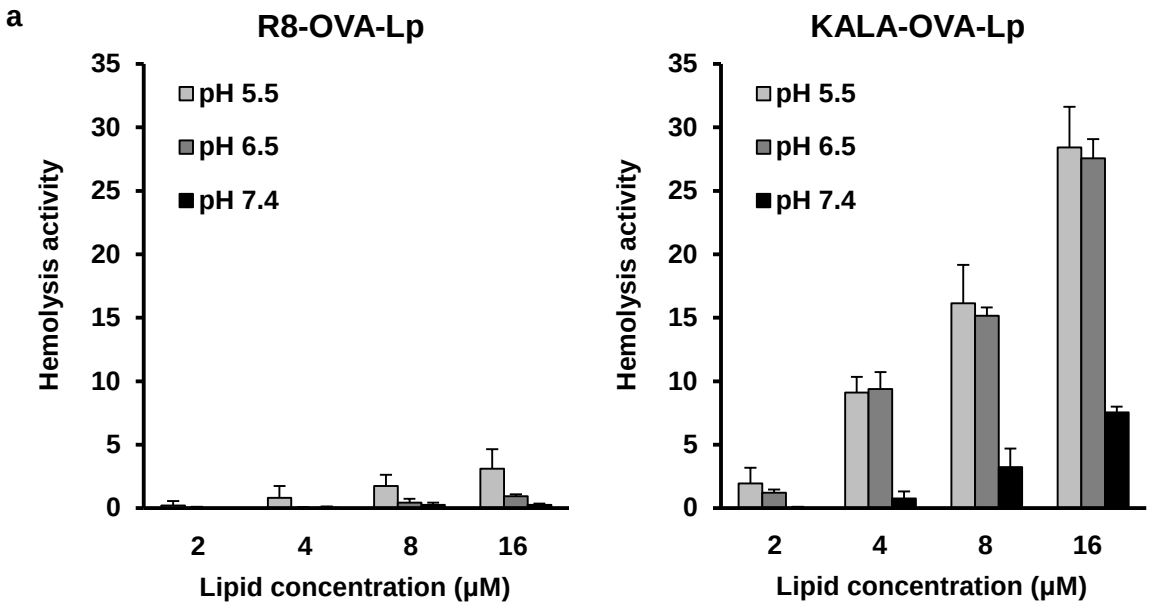

a
R8-OVA-Lp
KALA-OVA-Lp
Hemolysis activity
pH 5.5
pH 6.5
pH 7.4
0
5
10
15
20
25
30
35
2
4
8
16
Lipid concentration (μM)

Figure 5.

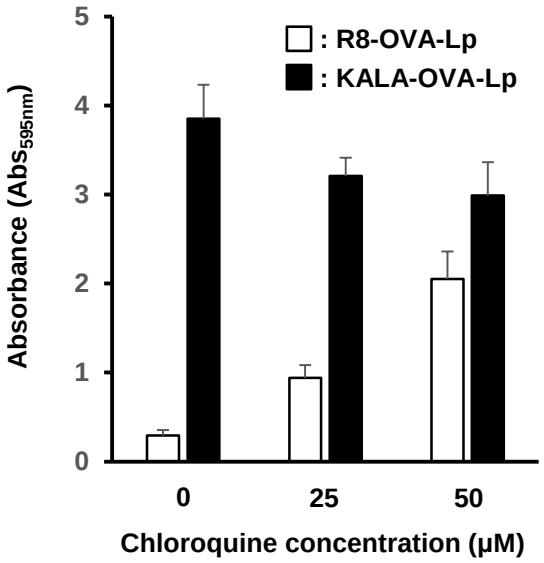

5
4
3
2
1
0
Absorbance (Abs595nm)
: R8-OVA-Lp
: KALA-OVA-Lp
0
25
50
Chloroquine concentration (μM)

Figure 6.

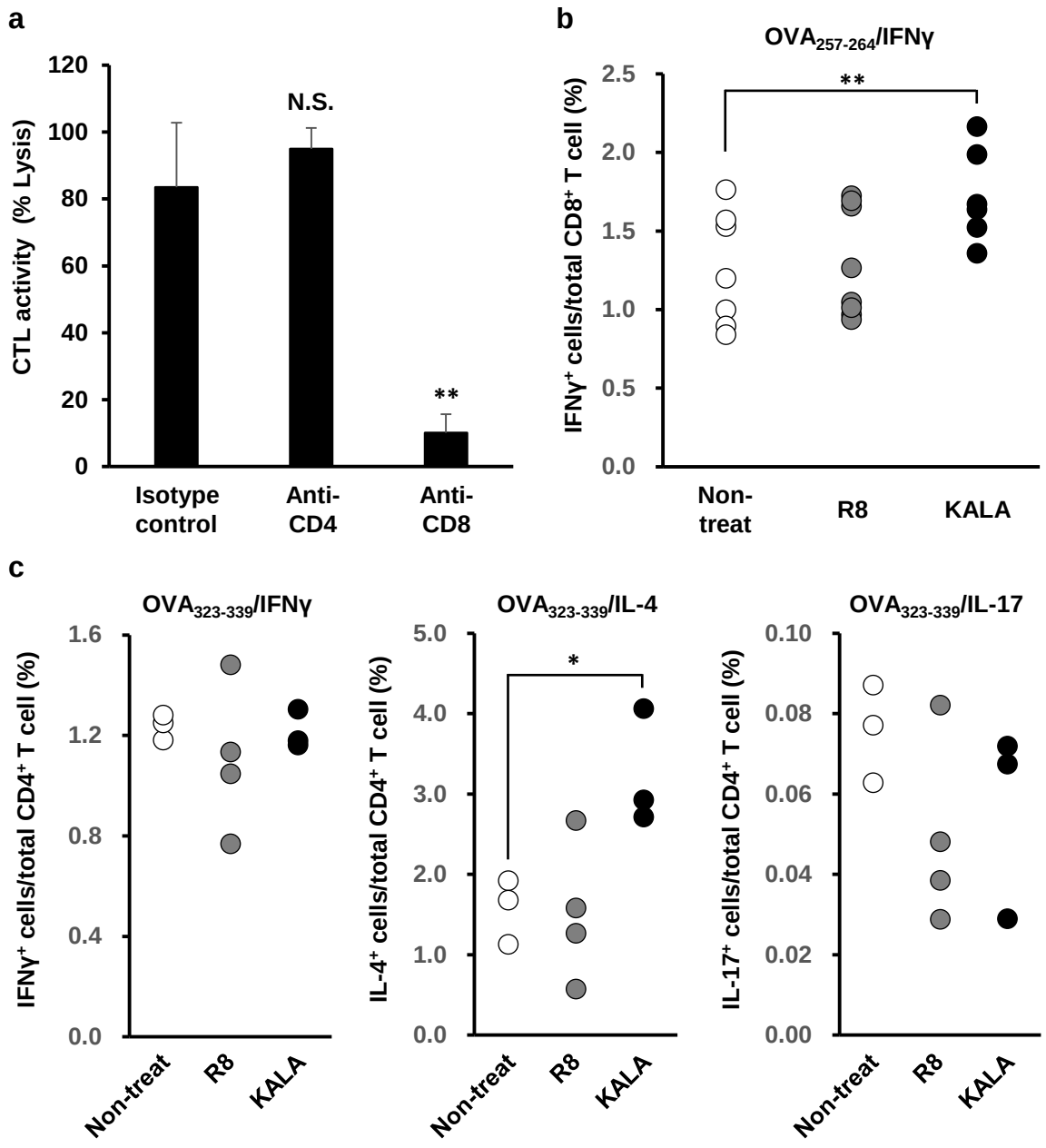
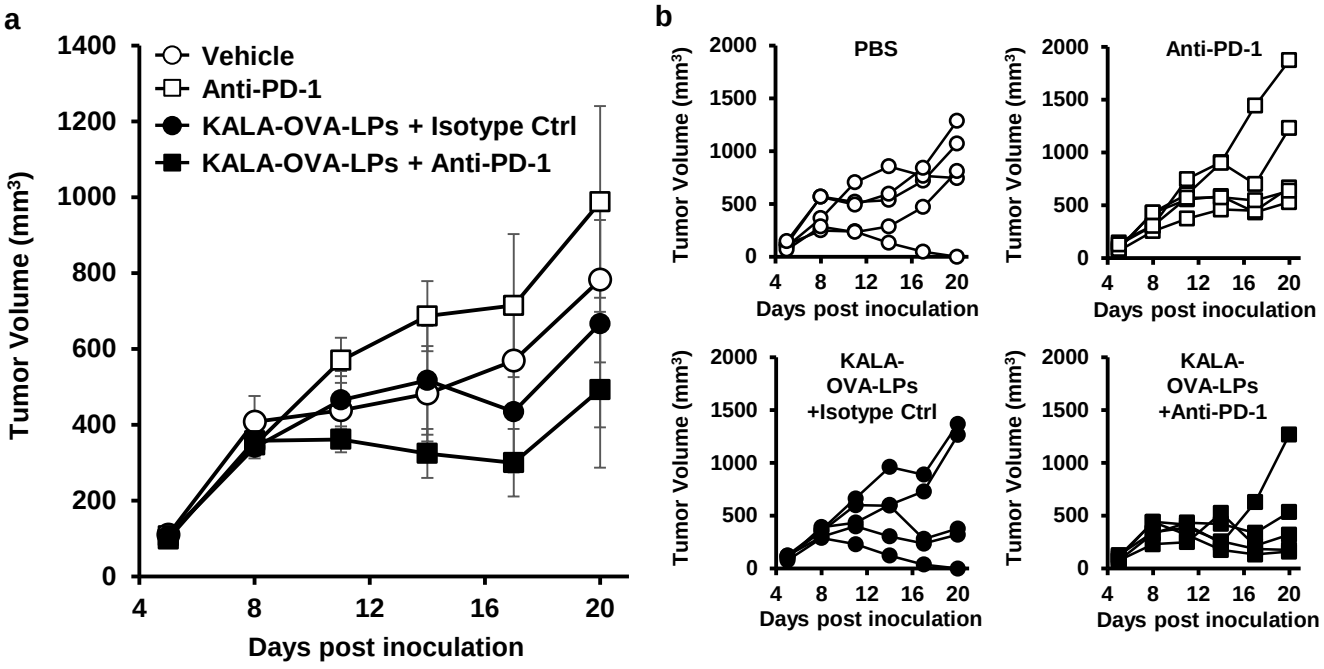

a
CTL activity (% Lysis)
120
100
80
60
40
20
0
N.S.
**
Isotype control
Anti-CD4
Anti-CD8
b
OVA257-264/IFNγ
IFNγ+ cells/total CD8+ T cell (%)
2.5
2.0
1.5
1.0
0.5
0.0
**
Non-treat
R8
KALA
c
OVA323-339/IFNγ
IFNγ+ cells/total CD4+ T cell (%)
1.6
1.2
0.8
0.4
0.0
Non-treat
R8
KALA
OVA323-339/IL-4
IL-4+ cells/total CD4+ T cell (%)
5.0
4.0
3.0
2.0
1.0
0.0
*
Non-treat
R8
KALA
OVA323-339/IL-17
IL-17+ cells/total CD4+ T cell (%)
0.10
0.08
0.06
0.04
0.02
0.00
Non-treat
R8
KALA

Table 1

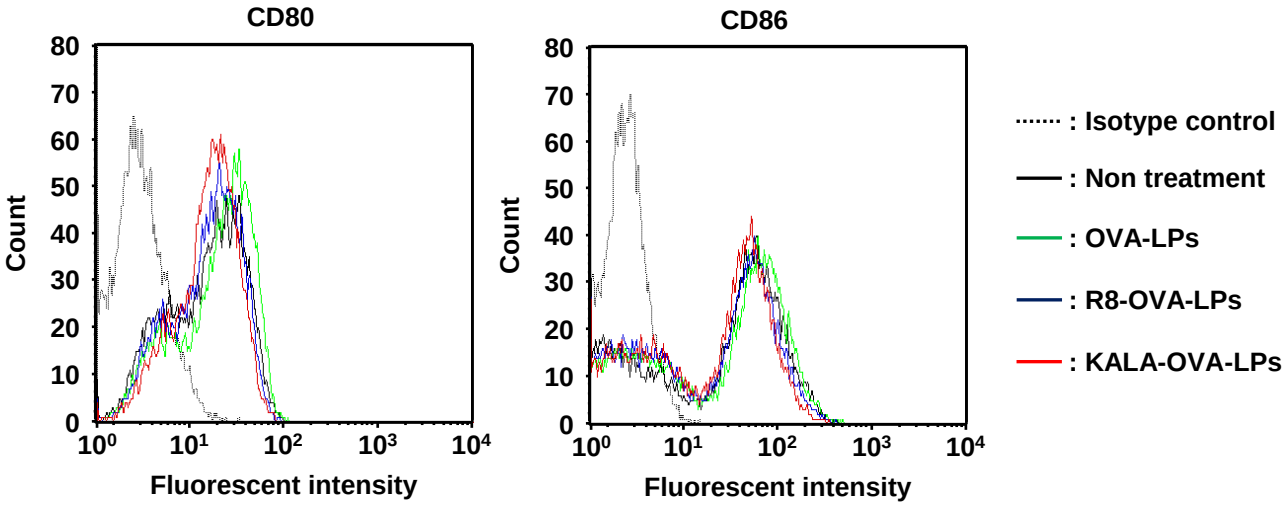
Liposome	Size (nm)	ζ-potential (mV)
OVA-Lp (DOPE)	158 ± 2	−19 ± 1
OVA-Lp (EPC)	162 ± 1	−37 ± 11
KALA-OVA-Lp (DOPE)	169 ± 4	50 ± 1
KALA-OVA-Lp (EPC)	185 ± 32	30 ± 1
R8-OVA-Lp (DOPE)	178 ± 4	52 ± 1
R8-OVA-Lp (EPC)	169 ± 2	52 ± 4

Supplementary Figure 1.



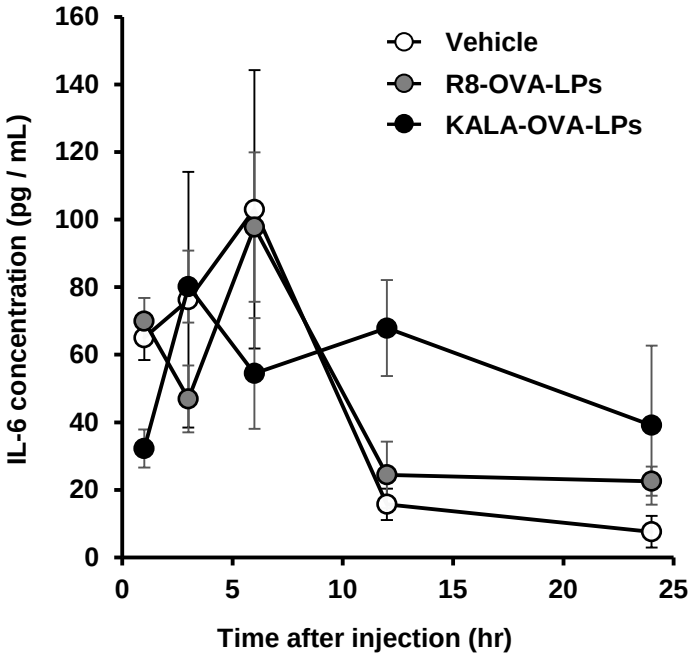
Supplementary Figure 1. Therapeutic anti-tumor effect combined with anti-PD-1 treatment
(a) C57BL/6 mice were inoculated with 8.0×10^5 cells of E.G7-OVA in the left flank. 5, 9, 13, 17 days after inoculation, mice were immunized subcutaneously with KALA-OVA-LPs at a dose of 25 μg OVA. Anti-PD-1 or Isotype Ctrl antibody was also administered intraperitoneally at a dose of 50 μg on every two days from first immunization. The tumor volume was measured up to 20 days after inoculation. The plots represent the mean ± SEM (n = 5). (b) Tumor volume of individual mouse of each group in the experiment (a).

Supplementary Figure 2.



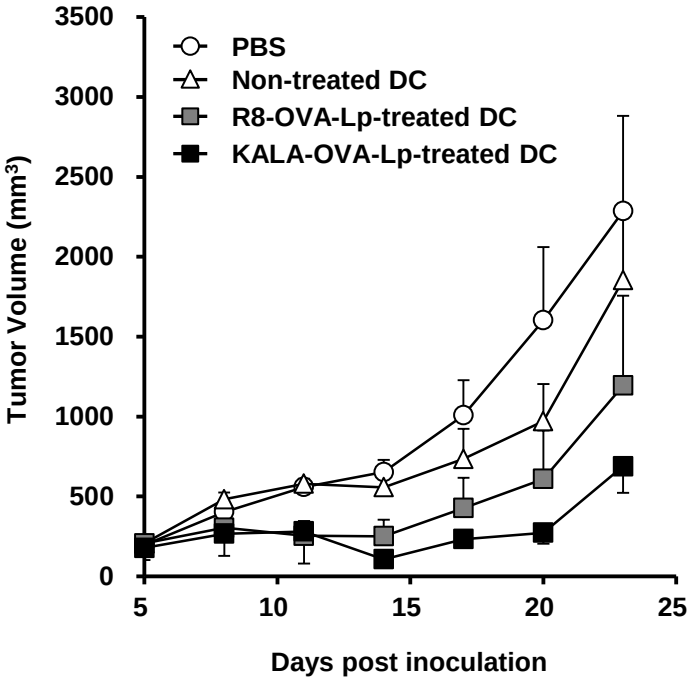
Supplementary Figure 2. CD80/86 expression in BMDCs that were treated with liposomes. BMDCs (1.0×10^6 cells) were treated with the KALA-OVA-LPs, the R8-OVA-LPs or the non-modified OVA-LPs at a lipid dose of 32 μM. After 18 hours, the BMDCs were recovered and stained by PE-labeled anti-mouse CD80 and CD86 (Biolegend).

Supplementary Figure 3.



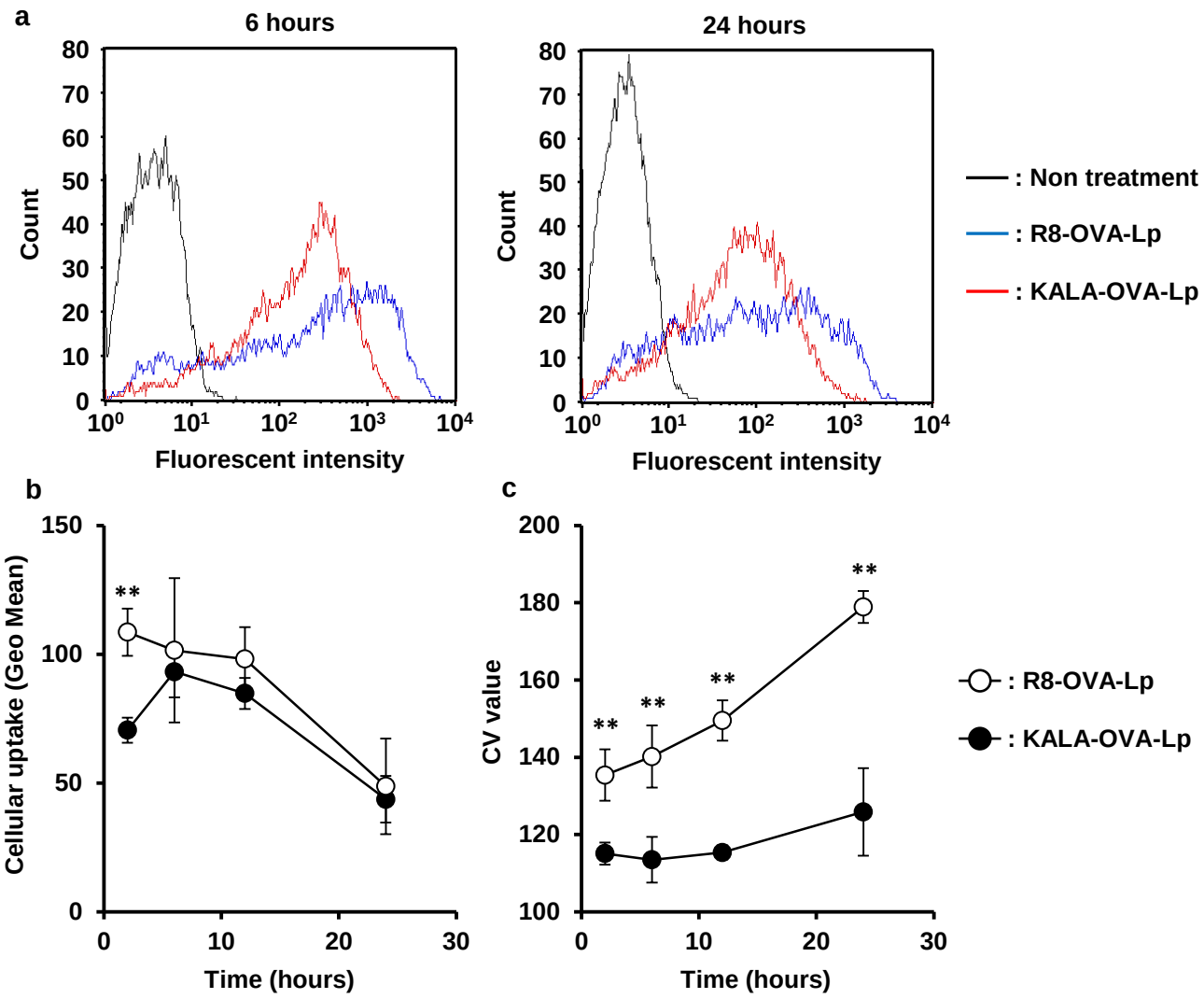
Supplementary Figure 3. IL-6 concentration in serum after liposome injection.
C57BL/6 mice were administered subcutaneously with KALA-OVA-LPs, R8-OVA-LPs at a dose of 25 μg OVA. Blood of these mice was collected at 1, 3, 6, 12 and 24 hrs after administration. Serum was separated from blood and the IL-6 concentration was measured by ELISA.

Supplementary Figure 4.



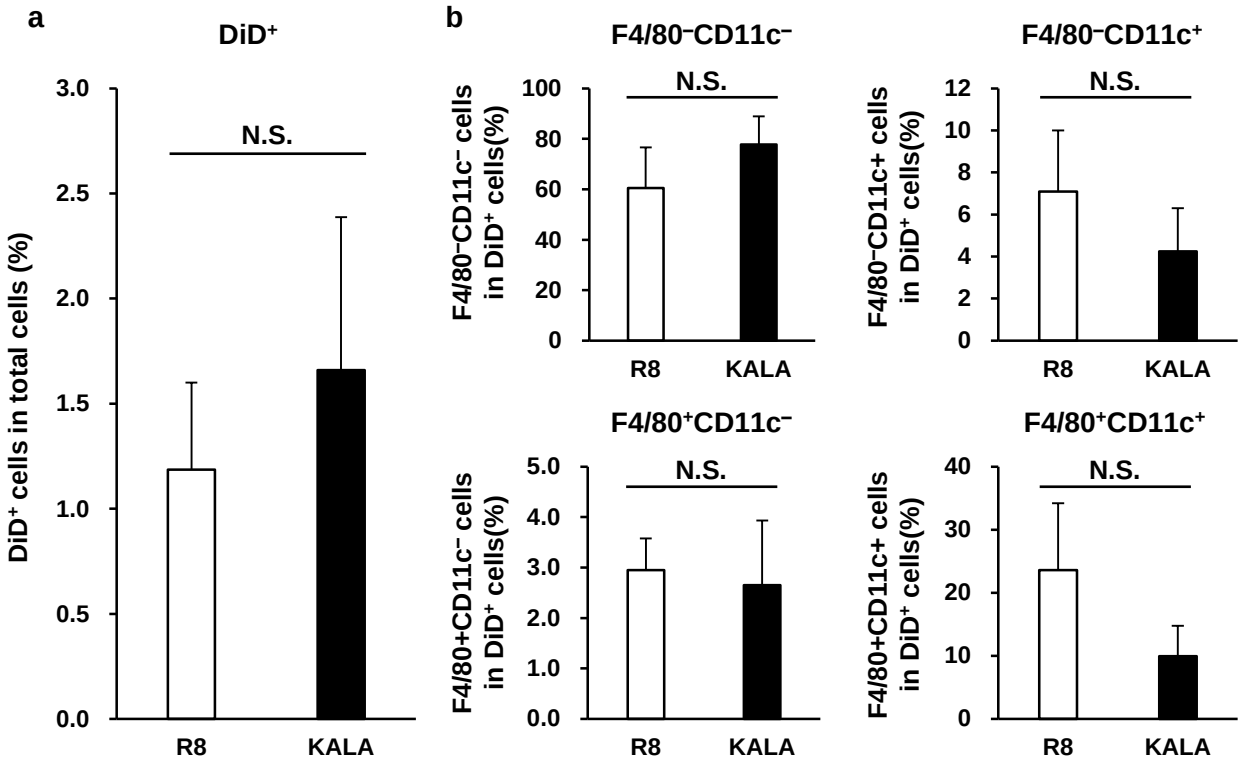
Supplementary Figure 4. *Ex vivo* anti-tumor experiment.
BMDCs (1.0×10^6 cells) were treated with the KALA-OVA-LPs or R8-OVA-LPs at a lipid dose of 32 μMd. After 6 hr incubation, the BMDCs were harvested. C57BL/6 mice were immunized with 5.0×10^5 cells of the harvested BMDCs treated with the KALA-OVA-LPs or the R8-OVA-LPs or non-treated BMDCs. At one week after immunization, mice were inoculated with 8.0×10^5 cells of E.G7-OVA in the left flank. Tumor volume was measured up to 23 days after inoculation. The plots represent the mean ± SEM (n = 4-5).

Supplementary figure 5.



Supplementary figure 5. Uptake of KALA-OVA-LPs and R8-OVA-LPs in BMDCs.
BMDCs were treated with KALA-OVA-LPs or R8-OVA-LPs encapsulating Alexa Flour 488-labeled OVA (25% of total OVA) at a lipid dose of 32 μM. After 2, 6, 12 or 24 hours, the BMDCs were recovered and measured the fluorescent intensity by flowcytometer. (a) Typical histogram of BMDCs treated with KALA-OVA-LPs or R8-OVA-LPs. (b) Average of fluorescence intensity (Geo mean, left) and coefficient variance (CV) value (right). Data are mean ± SD (n=3). Statistical analyses were performed by Student's t-test. **P < 0.01.

Supplementary figure 6.



Supplementary figure 6. Lymph node accumulation of KALA-OVA-LPs and R8-OBA-LPs
C57BL/6 mice were administered subcutaneously with KALA-OVA-LPs, R8-OVA-LP modified with 0.1 mol% DiD at a dose of 25 μg OVA in the both side flanks. After 24 hours, the draining lymph nodes (inguinal lymph nodes) were isolated and mashed. Nylon mesh-filtered cell suspension were analyzed by flow cytometry for uptake of the fluorescently-labeled liposomes and expression of F4/80 and CD11c. Data are mean + SEM (n=3). Statistical analyses were performed by Student's t-test. N.S.: Not significant.