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Liposome-Coupled Peptides Induce Long-Lived Memory CD8^{+} T Cells Without CD4^{+} T Cells

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Abstract

CD8^{+} T cells provide broad immunity to viruses, because they are able to recognize all types of viral proteins. Therefore, the development of vaccines capable of inducing long-lived memory CD8^{+} T cells is desired to prevent diseases, especially those for which no vaccines currently exist. However, in designing CD8^{+} T cell vaccines, the role of CD4^{+} T cells in the induction and maintenance of memory CD8^{+} T cells remains uncertain. In the present study, the necessity or not of CD4^{+} T cells in the induction and maintenance of memory CD8^{+} T cells was investigated in mice immunized with liposome-coupled CTL epitope peptides. When OVA-derived CTL epitope peptides were chemically coupled to the surfaces of liposomes and inoculated into mice, both primary and secondary CTL responses were successfully induced. The results were further confirmed in CD4^{+} T cell-eliminated mice, suggesting that CD4^{+} T cells were not required for the generation of memory CD8^{+} T cells in the case of immunization with liposome-coupled peptides. Thus, surface-linked liposomal antigens, capable of inducing long-lived memory CD8^{+} T cells without the contribution of CD4^{+} T cells, might be applicable for the development of vaccines to prevent viral infection, especially for those viruses that evade humoral immunity by varying their surface proteins, such as influenza viruses, HIV, HCV, SARS coronaviruses, and Ebola viruses.

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Introduction

It has been reported by numerous investigators that CD4^{+} T cells are essential for the maintenance of memory CD8^{+} T cells [1–5]. However, in the induction and maintenance of CD8^{+} memory T cells, different roles of CD4^{+} T cells have been described [6–9]. In the so-called "classical model", CD4^{+} T cells contribute to memory CD8^{+} T-cell generation indirectly via APCs [6]. Through the CD40-CD40L interaction between CD40L on CD4^{+} T cells and CD40 on APCs, CD4^{+} T cells "license" APCs for the induction of memory CD8^{+} T cells. As an alternative to this APC licensing model, Bourgeois et al. [7] provided evidence demonstrating that CD4^{+} T cells contribute directly to CD8^{+} T cells through CD40 on CD8^{+} T cells, rather than indirectly via APCs. However, these findings were countered by studies in which long-lived CD8^{+} memory T cells were generated in the absence of CD40 expression on CD8^{+} T cells [8,9]. In addition, as for the role of CD40-CD40L interaction in the induction of memory CD8^{+} T cells, Hernandez et al. [10] reported that CD8^{+} T cells themselves provided CD40L in order to license APCs for the induction of memory CD8^{+} T cells. In their scenario, although the CD40-CD40L interaction between T cells and DCs is indispensable for the induction of memory CD8^{+} T cells, CD4^{+} T cells are not necessarily involved. Thus, the research so far has not resolved the role of CD4^{+} T cells in the induction and maintenance of memory CD8^{+} T cells, although resolving this issue is a critical step in designing better vaccination and immunotherapeutic strategies.

Upon natural infection, the host responds by inducing humoral and cellular immunity against the pathogen. Humoral immune responses are represented by the production of antibodies that bind to the surfaces of bacteria and viruses, whereas cellular immune responses mediate immunity to intracellular pathogens. In general, extracellular antigens are presented via MHC class II molecules to CD4^{+} T cells, whereas intracellular antigens are presented via MHC class I molecules to CD8^{+} T cells. To induce antigen-specific CTL, antigens must be loaded onto the class I MHC processing pathway in APCs via cross-presentation [11]. In the cross-presentation, exogenous proteins cross over to the endogenous pathway to gain access to MHC class I molecules. Using this phenomenon, a generation of antigen-specific CTL responses might be useful in the development of vaccines that can prevent viral diseases. However, the currently approved alum adjuvant, which was first described by Glenny et al. [12] in 1926 and until today remains the only adjuvant approved for clinical use, is known to be effective only for the induction of humoral immunity, not for the induction of cell-mediated immunity [13–16]. Consequently, the development of a novel vaccine adjuvant is essential for the induction of cell-mediated immunity.

We previously reported that surface-coupled liposomal antigens could be presented by APCs to CD8^{+} T cells via MHC class I molecules if certain lipid components were chosen for the liposomes [17]. This antigen preparation was expected to be applicable for the development of tumor vaccines to induce antitumor responses and for the development of viral vaccines to induce virus-specific CTLs that effectively eliminate virus-infected cells [18]. Since the liposomal conjugates induced CTLs efficiently when CTL epitope peptides were coupled to the surfaces of liposomes [17], the liposomal conjugatesare expected to be applicable for the development of CTL-based peptide vaccines. In the development of peptide vaccines, it is essential to know whether a T helper epitope peptide is necessary for the induction of long-lived memory $CD8^+$ T cells, an important step in vaccine preparation. This study was aimed at evaluating the role of $CD4^+$ T cells in the induction of long-lived memory $CD8^+$ T cells by liposome-coupled peptides.

Results

Induction of antigen-specific primary $CD8^+$ T cells and CTLs in mice by $OVA_{257-264}$-liposome conjugates

Mice were immunized with $OVA_{257-264}$-liposome conjugates in the presence of CpG as described in Materials and Methods. A significant induction of CTL specific for $OVA_{257-264}$ was observed on day 4 and a complete cell killing was observed as early as 5 days after the immunization (Figure 1). Therefore, in the following experiments, primary CTL responses were monitored at 7 days after immunization. Mice were then immunized with serially diluted solution of $OVA_{257-264}$-liposome conjugates containing 0.3 (8×) to 2.4 μg (1×) of peptides or $OVA_{257-264}$ solution that contained equal amounts of peptides as those in liposomal conjugates. Although $OVA_{257-264}$-liposome and $OVA_{257-264}$ solution seemed to induce a comparable level of T-cell cytokine production at the highest dose (2.4 μg/injection), a dose-dependent decrease was observed in mice immunized with $OVA_{257-264}$ solution but not in mice immunized with $OVA_{257-264}$-liposome, suggesting that $OVA_{257-264}$-liposome was more effective than $OVA_{257-264}$ solution in the induction of antigen-specific $CD8^+$ T cell cytokine production (Figure 2A). Similar results were observed in T cell cytokine production; a dose of $OVA_{257-264}$-liposome as low as 0.6 μg/mouse (4× dilution) induced a perfect killing as assayed by *in vivo* CTL assay, while $OVA_{257-264}$ solution induced only a partial killing even at the highest dose (Figure 2B).

Secondary CTL response in mice immunized with $OVA_{257-264}$-liposome

Induction of secondary CTL responses in mice immunized with $OVA_{257-264}$-liposome was further investigated. Mice were immunized with 50 μl of $OVA_{257-264}$-liposome and 2, 4, 8, 16, and 20

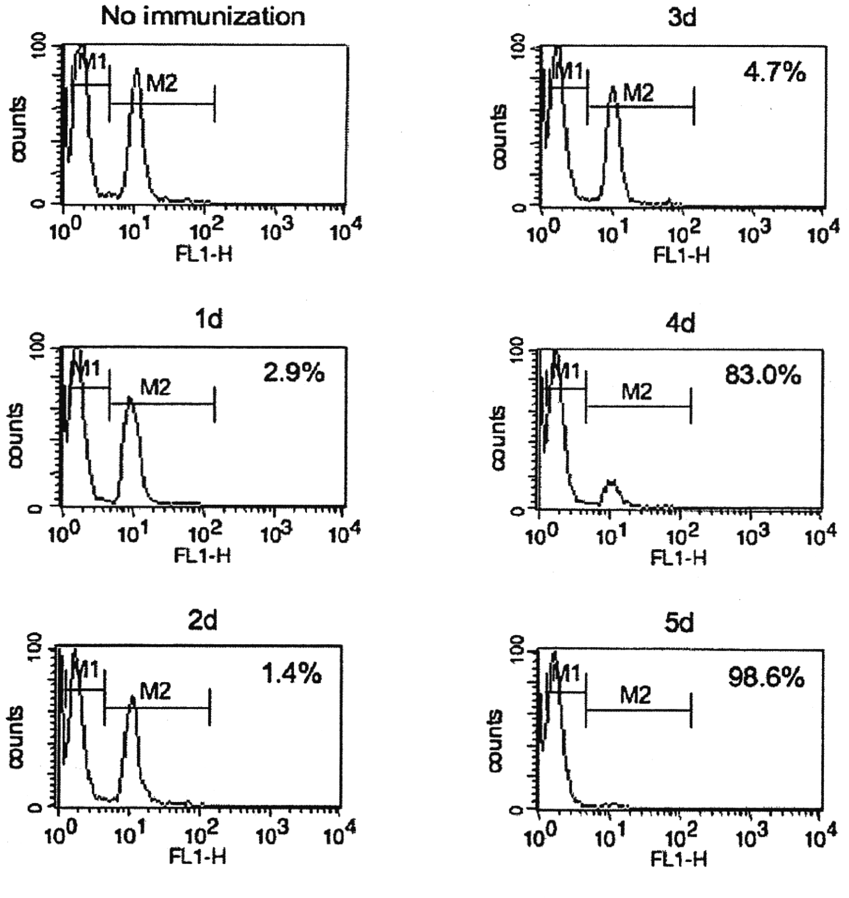

Figure 1. Kinetics of primary CTL response induced by $OVA_{257-264}$-liposome conjugates. Mice were immunized with 50 μl of $OVA_{257-264}$-liposome in the presence of 5 μg CpG; one to 5 days later, an *in vivo* CTL assay was performed as described in Materials and Methods. The numbers for each time period indicate percentages of target cells killed. Data are representative of three individual mice in each group for which similar results were obtained.
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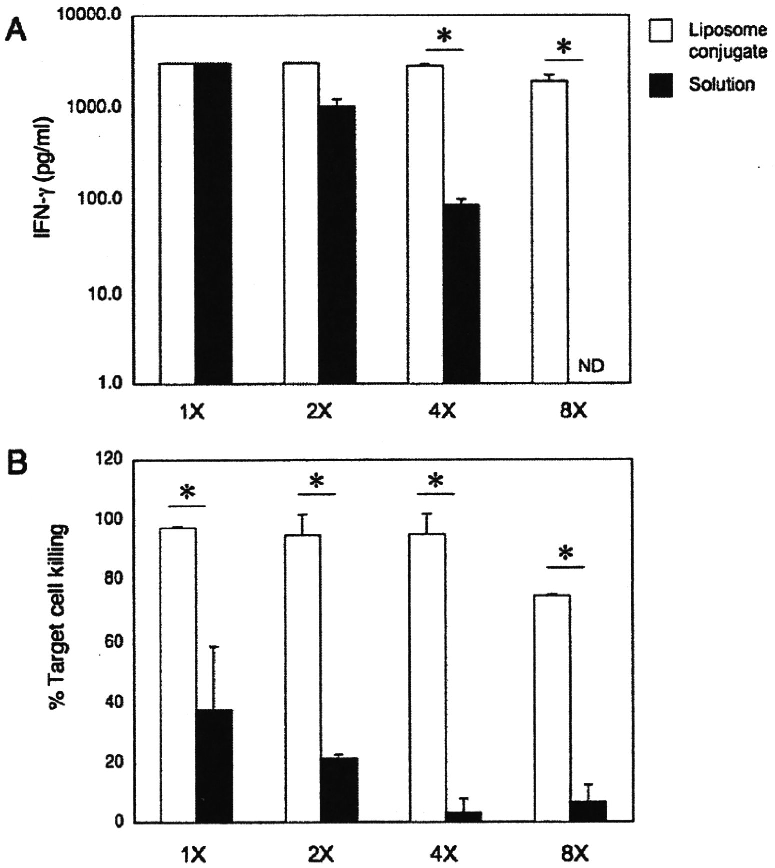

Figure 2. Dose-response of cytokine production by CD8$^+$ T cell and CTL induction in mice immunized with OVA$_{257-264}$-liposome or with OVA$_{257-264}$ solution. A serial two-fold dilution of OVA$_{257-264}$-liposome (open box) and OVA$_{257-264}$ solution (closed box) were made in PBS, and mice were immunized with the diluents in the presence of 5 µg CpG. OVA$_{257-264}$ solution containing equal amounts of peptides as those in OVA$_{257-264}$-liposome. One week after the immunization, IFN-γ production by CD8$^+$ T cells (**A**) and the CTL response (**B**) were monitored as described in Materials and Methods. Data represent means and SE of three mice per group. *, significant difference ($p>0.01$).
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weeks later, the mice received a booster injection with OVA. Three days after the booster injection, OVA$_{257-264}$-specific cell killing was monitored. As shown in Figure 3, a complete cell killing was observed at 2 weeks after the immunization without a booster injection and, up to 20 weeks after the immunization, a significant recall response was observed upon booster injection with OVA. Inoculation of naive mice with the same dose of OVA as the booster injection ("No imm." in Figure 3) did not induce a detectable CTL response. Interestingly, a significant recall response was observed even at 20 weeks when the primary CTL response was nearly undetectable. An antigen-specific CD8$^+$ T-cell proliferation assay further confirmed the results; as shown in Figure 4, CD8$^+$ T cells of mice immunized with OVA$_{257-264}$-liposome significantly proliferated upon *in vitro* stimulation with OVA even 20 weeks after immunization.

Effect of in vivo elimination with CD4$^+$ T cells on the induction of long-lived memory CD8$^+$ T cells by OVA$_{257-264}$-liposome conjugates

To eliminate CD4$^+$ T cells, mice were inoculated with GK1.5 as described in Materials and Methods, and immunized with OVA$_{257-264}$-liposome. As shown in Figure 5, *in vivo* elimination with CD4$^+$ T cells affected neither for primary (Figure 5A) nor for secondary (Figure 5B) CTL responses; even at 20 weeks after the immunization, a significant recall response, comparable to that in normal mice, was observed in mice from which CD4$^+$ T cells had been eliminated.

Discussion

In the present study, the role of CD4$^+$ T cells in the induction and maintenance of memory CD8$^+$ T cells was evaluated in mice immunized with liposome-coupled CTL epitope peptides. Although the inclusion of CpG, a ligand of TLR-9, was needed for the induction of the primary CTL response by OVA$_{257-264}$-liposome, CD4$^+$ T cells were not required in either primary or secondary response, since long-lived memory CD8$^+$ T cells were readily induced only by immunization with CTL epitope peptides coupled to liposomes (Figures 3 and 4). This finding was further confirmed in CD4$^+$ T cell-depleted mice (Figure 5). These results are in agreement with those reported previously by numerous investigators that CD4$^+$ T cells are dispensable for the primary expansion of CD8$^+$ T cells and their differentiation into cytotoxic

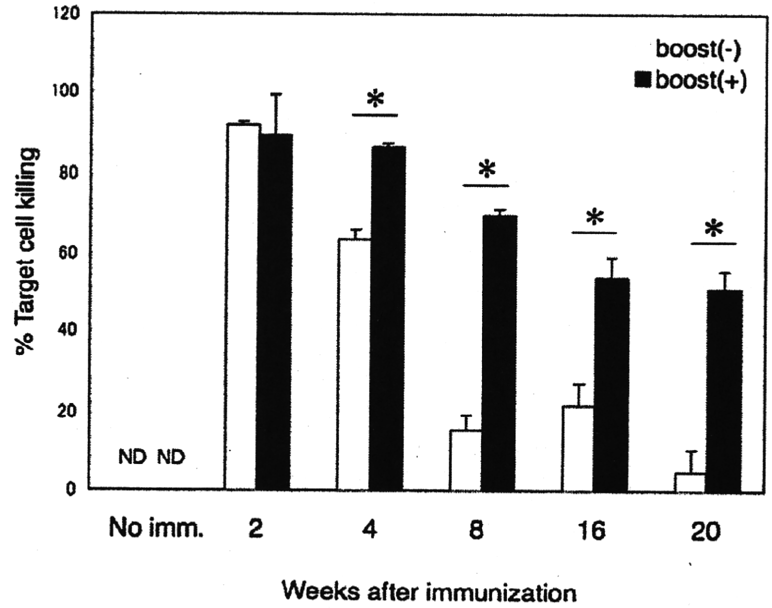

Figure 3. Secondary CTL response in mice immunized with $OVA_{257\text{-}264}$-liposome. Mice were immunized with 50 µl of $OVA_{257\text{-}264}$-liposome in the presence of 5 µg CpG, and 2, 4, 8, 16, and 20 weeks later, they received a booster ip injection with 200 µl of 1 mg/ml OVA in PBS (closed box) or no booster injection (open box). Three days after the booster injection, *in vivo* CTL assay was performed. Data represent mean percentages of cells killed and SEs of three mice per group. ND, not detected. *, significant difference ($p>0.01$).
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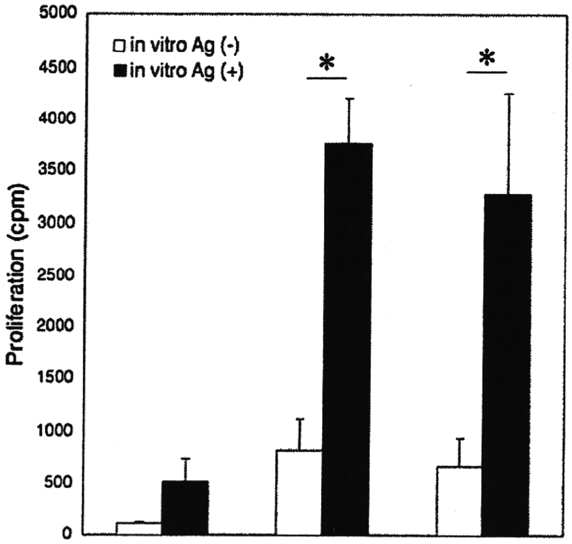

Figure 4. Antigen-specific $CD8^+$ T-cell proliferation assay. Mice were immunized with $OVA_{257\text{-}264}$-liposome and 1 week or 20 weeks later, $CD8^+$ T cells of the immunized mice were cultured in the presence (closed box) or absence (open box) of OVA as described in Materials and Methods. Data represents mean ^3H- thymidine incorporation and SE of triplicate cultures. *, significant difference ($p>0.01$).
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effectors [2,3,5]. However, most of these researchers have claimed that secondary CTL expansion is wholly dependent on the presence of T helper cells during, but not after, priming [1–5].

We previously reported that surface-linked liposomal antigens induced IgE-selective unresponsiveness [19]. The results were consistent even when different coupling procedures for the antigens with the liposomes were employed [20]. During the course of an investigation intended to clarify the mechanism of IgE-selective unresponsiveness induced by surface-coupled liposomal antigens, we discovered an alternative approach to regulating the production of IgE, one that is independent of the activity of T cells [21]. Immunization of mice with OVA-liposome conjugates induced IgE-selective unresponsiveness without apparent Th1 polarization. Neither interleukin-12 (IL-12), IL-10, nor $CD8^+$ T cells participated in the regulation. Further, $CD4^+$ T cells of mice immunized with OVA-liposome were capable of inducing antigen-specific IgE synthesis in athymic nude mice immunized with alum-adsorbed OVA. On the other hand, immunization of the recipient mice with OVA-liposome did not induce anti-OVA IgE production, even when $CD4^+$ T cells of mice immunized with alum-adsorbed OVA were transferred. In the secondary immune response, OVA-liposomes enhanced anti-OVA IgG antibody production but did not enhance ongoing IgE production, suggesting that the IgE-selective unresponsiveness induced by the liposomal antigen involved direct effects on IgE but not IgG switching *in vivo*. These results suggest the role of an alternative mechanism, one not involving T cells, in the regulation of IgE synthesis, and raise the possibility that the surface-linked liposomal antigens are potentially applicable for the development of novel vaccines with minimal induction of IgE synthesis. Moreover, given the relatively low allergic response to and increased antigenicity of the allergen, this form of antigen preparation would be applicable for allergen immunotherapy [22].

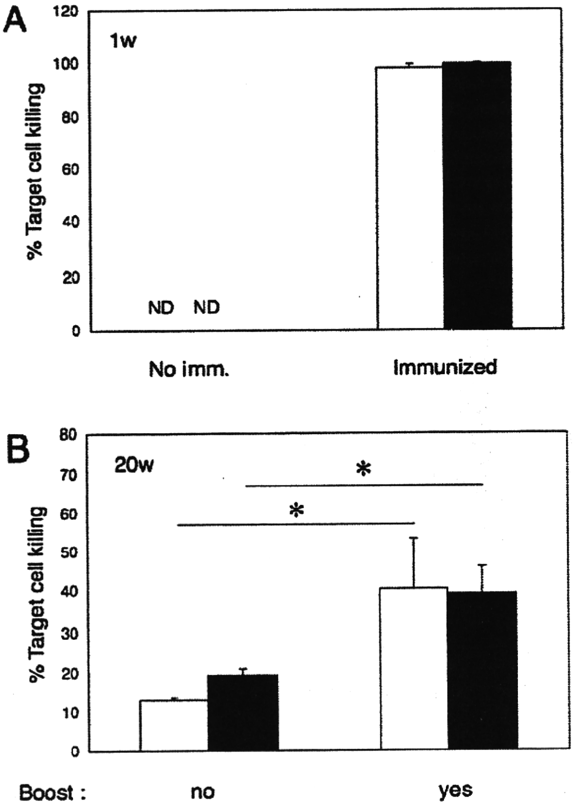

Figure 5. Effect of *in vivo* elimination of CD4$^+$ T cells on the induction of primary and secondary CTL responses by OVA$_{257-264}$-liposomes. Mice with (closed box) or without (open box) CD4$^+$ T-cell elimination were immunized with 50 µl of OVA$_{257-264}$-liposome solution in the presence of 5 µg CpG, and CTL induction was monitored. **A**, CTL response 1 week after immunization. **B**, CTL response 20 weeks after immunization with or without booster injection. *In vivo* CTL assay was performed 3 days after the booster injection. Data represent mean percent killing and SE of three mice per group. ND, not detected. *, significant difference ($p>0.01$).
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The potential usefulness of surface-linked liposomal antigens for application to vaccine development was further investigated. During the course of this investigation, a significant difference was observed in the recognition of liposomal antigens by antigen-presenting cells (APCs) between liposomes with different lipid components [23], and this difference was closely correlated with the adjuvant activity of liposomes [24]. In addition to this "quantitative" difference between liposomes with different lipid components, a "qualitative" difference (i.e., different abilities to induce cross-presentation) was also observed between liposomes with different lipid components [17]. Although the precise mechanism underlying this difference is currently unclear, the significant difference in membrane mobility observed between these liposomes [24] might affect their ability to induce cross-presentation. Thus, by utilizing their ability to induce cross-presentation, surface-linked liposomal antigens could be used to develop virus vaccines that induce a cytotoxic T-cell (CTL) response, as well as tumor vaccine preparations that present tumor antigens to APCs and induce effective antitumor responses [18].

Regarding the necessity of CD4$^+$ T cells in the generation of memory CD8$^+$ T cells, the results of the present study differed from those reported previously [1-5]. The difference in these findings may be due to differences in how mice were primed with antigens; in most of the studies reported previously, mice were primed by infecting viruses, such as LCMV [1,3,5], H3N2 influenza virus [2], and recombinant vaccinia virus [4], whereas in the present study, mice were immunized with OVA-derived CTL epitope peptides. Perhaps the difference in the requirements of CD4$^+$ T cells observed among those studies [1–5] and the present study was due to the difference in the efficiency of inducing the presentation of the immunodominant CTL epitope by APCs. In general, only ~1/2000 of the peptides in a foreign antigen expressed by an appropriate APC achieve immunodominant status with a given class I allele [25]. However, in the present study, immunization with OVA$_{257-264}$-liposome successfully induced both primary and secondary CTL responses without the presence of CD4$^+$ T cells (Figures 2 to 5). In addition, it was reported previously that antigens coupled to the surface of liposomes are recognized effectively by APCs and presented to T cells [24]. Therefore, although the TLR-ligand (CpG, in the present study) was necessary to mimic viral infection in order to induce CTL responses in the immunization with liposome-coupled peptides, CD4$^+$ T cells were not required for the induction and maintenance of CD8$^+$ memory T cells.

There is considerable interest in developing vaccines that elicit effective antiviral CD8$^+$ T cell responses [26] against a variety of viruses, such as HIV [27], HCV [28], and SARS coronavirus [29]. For this purpose, the utilization of the immunodominant CTL epitope would be more effective than the use of an attenuated, inactivated, or subunit vaccine in the development of virus vaccines to elicit effective antiviral CD8$^+$ T cell responses. For example, although the risk of a major global pandemic of avian influenza has created widespread concern, vaccines designed to induce antibodies against H5 haemagglutinin are expected to possess little or no efficacy, given the high rate of diversification of H5N1 strains due to the antigenic drift caused by point mutation of genes [30–33]. On the other hand, it is known that cytotoxic T cells specific for the internal proteins NP and M1 show high cross-reactivity between strains and between subtypes, reflecting high conservation of the internal proteins [34–37]. In addition, Lee et al. [38] recently reported that people who have not been exposed to H5N1 viruses have cross-reactive CD8$^+$ T cell memory to a wide range of H5N1 peptides. Therefore, these peptides are expected to be used to add a CD8$^+$ T cell component to current antibody-focused vaccine strategies with a view to reducing the impact of infection with novel influenza A viruses [39]. Epstein et al. [40] studied DNA vaccination in mice with plasmids expressing conserved nucleoprotein (NP) and matrix (M) from an H1N1 virus. However, the DNA vaccination alone protected poorly against a highly virulent strain of H5N1 influenza viruses.

Recently, we reported that peptides derived from the internal NP protein of the H3N2 influenza virus, chemically coupled to the surface of liposomes, induced antigen-specific CTLs and successfully inhibited the growth of H3N2 influenza virus in the lung [41]. More recently, we determined human HLA class I-restricted, immunodominant CTL epitopes derived from internal proteins of H5N1 influenza viruses [42]. Similar to those results reported previously [34–37,43], most of the CTL epitopes determined were well conserved and were identical with those involved in H1N1 and H3N2 influenza viruses. The combined use of these CTL epitope peptides, common to influenza viruses, and the surface-linked liposomal antigens which induce long-lived memory CD8$^+$ T cells without CD4$^+$ T cell help, was demonstrated to be applicable for the development of a CTL-based influenza vaccine that is capable of inducing protection against heterosubtypic influenza viruses [42].

Taken together, these results suggest that surface-linked liposomal antigens might be applicable for the development of CTL-based vaccines to induce long-term prevention against infection with viruses other than influenza viruses, especially for those viruses that evade humoral immunity by varying their surface proteins, such as HIV, HCV, and SARS coronaviruses.

Materials and Methods

Mice

CBF1 mice (5–6 wk of age) were purchased from SLC (Shizuoka, Japan). All mice were maintained under specific pathogen-free conditions. Experiments in the present study were approved (permit numbers 208021 and 209082) by the Animal Research Committee of National Institute of Infectious Diseases, Tokyo, Japan and the mice were handled according to international guidelines for experiments with animals.

Chemicals

All phospholipids were obtained from NOF Co. (Tokyo, Japan). Reagent grades of cholesterol were purchased from Wako Pure Chemicals (Osaka, Japan).

Antigens and Reagents

Ovalbumin (OVA, grade VII) was purchased from Sigma-Aldrich. Mouse MHC class-I (K^b)-binding peptides $OVA_{257-264}$ (SIINFEKL) were obtained from Operon Biotechnologies (Tokyo, Japan). Synthetic CpG ODN (5002: TCCATGACGTTCTT-GATGTT), phosphorothioate-protected to avoid nuclease-dependent degradation, was purchased from Invitrogen.

Liposomes

The liposomes used in this study are provided by NOF corporation (Tokyo, Japan). They consisted of dioleoyl phosphatidylcholine (DOPC), dioleoyl phosphatidyl ethanolamine (DOPE), dioleoyl phosphatidyl glycerol (DOPG), and cholesterol in a 4:3:2:7 molar ratio. The crude liposome solution was passed through a membrane filter (Nucleopore polycarbonate filter; Coster) with a pore size of 0.2 μm.

Coupling of OVA peptides to liposomes

Liposomal conjugates with OVA peptides were prepared essentially in the same way as described previously [17] via disuccinimidyl suberate (DSS). Briefly, a mixture of 10 ml of anhydrous chloroform solution containing 0.136 mM DOPE and 24 μl of TEA was added in drops to 26.6 ml of anhydrous chloroform solution containing 0.681 mM DSS and stirred for 5 h at 40°C. The solvent was evaporated under reduced pressure, and 18 ml of a 2:1 mixture of ethyl acetate and tetrahydrofuran was added to dissolve the residue. Then, 36 ml of 100-mM sodium phosphate (pH 5.5) and 90 ml of saturated NaCl aqueous solution were added to the solution, shaken for 1 min, and allowed to separate. To remove undesirable materials, the upper layer was washed with the same buffer and, after evaporation of the solvent, 3 ml of acetone was added to dissolve the residue. One hundred ml of ice-cold acetone was added in drops and kept on ice for 30 min to precipitate. Crystals were collected and dissolved in 5 ml of chloroform. After evaporation, 34.4 mg of DOPE-DSS was obtained. Then, 0.18 mM DOPC, 0.03 mM DOPE-DSS, 0.21 mM cholesterol, and 0.06 mM DOPG were dissolved in 10 ml of chloroform/methanol. The solvent was removed under reduced pressure and 5.8 ml of phosphate buffer (pH 7.2) was added to make a 4.8% lipid suspension. The vesicle dispersion was extruded through a 0.2-μm polycarbonate filter to adjust the liposome size. A 2-ml suspension of DSS-introduced liposome and 0.5 ml of 5-mg/ml OVA peptide solution were mixed and stirred for 3 days at 4°C. The liposome-coupled- and uncoupled peptides were separated as described above using CL-4B column chromatography. The resulting solution of $OVA_{257-264}$-liposome conjugates contained 47 μg/ml of peptides as assessed by amino-acid quantitative analysis done by Toray Research Center (Kanagawa, Japan).

Immunization

All the mice were immunized with indicated doses of $OVA_{257-264}$-liposome conjugates via subctaneous injection in the presence of 5 μg/mouse CpG. For the booster immunization, the mice were immunized intraperitoneally (ip) with 200 μl of 1-mg/ml OVA in PBS solution.

In vivo elimination of $CD4^+$ T cells

For the *in vivo* elimination of $CD4^+$ T cells, mice received weekly ip injection with 0.5 mg of GK1.5, a monoclonal anti-CD4 antibody, throughout the experimental period. This treatment resulted in a >99% decrease in the number of $CD4^+$ T cells in the spleen and lymph nodes as determined by fluorescence-activated cell sorter (FACS) analysis.

In vivo cytotoxicity assay

Spleen cells of naive CBF1 mice were labeled with either 0.5 μM (dull) or 5 μM (bright) CFSE for 15 min at 37°C using a Cell Trace CFSE cell proliferation kit (Molecular Probes, Eugene, OR) and washed twice with ice-cold PBS. CFSE-bright cells were subsequently pulsed with 0.5 μg/ml of $OVA_{257-264}$ for 90 min at 37°C. CFSE-bright cells and CFSE-dull cells were mixed at a 1:1 ratio, and then a total of 1×10^6 cells was injected i.v. into the indicated group of mice. Twenty hours later, spleen cells were harvested from each mouse and analyzed by using FACSCalibur (Becton Dickinson, Mountain View, CA).

Cell culture

All incubations were performed in RPMI-1640 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS (HyClone), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen).

Preparation of dendritic cells (DC) and $CD8^+$ T cells

DCs and $CD8^+$ T cells were obtained from spleen cells of CBF1 mice using the magnetic cell sorter system MACS according to the manufacturer's protocol using anti-CD11c and anti-CD8 antibody-coated microbeads (Miltenyi Biotec), respectively. $CD8^+$ T cells and DCs were suspended in RPMI-1640 containing 10% FCS at cell densities of 2×10^6/ml and 8×10^5/ml, respectively. The $CD8^+$ T cell suspension was plated at 250 μl per well onto 48-well culture plates (No. 3047; BD Biosciences), and 250 μl of DC suspension and 500 μl of 40 μM $OVA_{257-264}$ solution in the same medium were added to the plates. After incubation in a CO_2 incubator for 5 days, the culture supernatants were collected and assayed for the concentration of IFN-γ.

Cytokine assays

IFN-γ in the culture supernatant was measured using the Biotrak mouse ELISA system (GE Healthcare, UK). All test samples were assayed in duplicate, and the SE in each test was always less than 5% of the mean value.

T cell proliferation assay

Splenic CD8$^+$ T cells (5×10^5 cells/well) of immunized mice and whole spleen cells (1×10^5 cells/well) of 25 Gy-irradiated naïve mice were cultured in 96-well plates for 4 days in the presence (closed box) or absence (open box) of 20 µM OVA. The cells were pulsed with 1.25 µCi (0.046 MBq) [^{3}H]-thymidine (PerkinElmer) for the final 6 hours of the culture, and, after harvesting, cell proliferation was monitored using TopCount (PerkinElmer).

Statistical analysis

Student's *t* test was employed for the statistical analysis.

Author Contributions

Conceived and designed the experiments: TU. Performed the experiments: MT YT TK. Analyzed the data: MT TU. Contributed reagents/materials/analysis tools: MT YT. Wrote the paper: TU. N/A.

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Liposome-Coupled Antigens Are Internalized by Antigen-Presenting Cells via Pinocytosis and Cross-Presented to $CD8^+$ T Cells

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Abstract

We have previously demonstrated that antigens chemically coupled to the surface of liposomes consisting of unsaturated fatty acids were cross-presented by antigen-presenting cells (APCs) to $CD8^+$ T cells, and that this process resulted in the induction of antigen-specific cytotoxic T lymphocytes. In the present study, the mechanism by which the liposome-coupled antigens were cross-presented to $CD8^+$ T cells by APCs was investigated. Confocal laser scanning microscopic analysis demonstrated that antigens coupled to the surface of unsaturated-fatty-acid-based liposomes received processing at both MHC class I and class II compartments, while most of the antigens coupled to the surface of saturated-fatty-acid-based liposomes received processing at the class II compartment. In addition, flow cytometric analysis demonstrated that antigens coupled to the surface of unsaturated-fatty-acid-liposomes were taken up by APCs even in a 4°C environment; this was not true of saturated-fatty-acid-liposomes. When two kinds of inhibitors, dimethylamiloride (DMA) and cytochalasin B, which inhibit pinocytosis and phagocytosis by APCs, respectively, were added to the culture of APCs prior to the antigen pulse, DMA but not cytochalasin B significantly reduced uptake of liposome-coupled antigens. Further analysis of intracellular trafficking of liposomal antigens using confocal laser scanning microscopy revealed that a portion of liposome-coupled antigens taken up by APCs were delivered to the lysosome compartment. In agreement with the reduction of antigen uptake by APCs, antigen presentation by APCs was significantly inhibited by DMA, and resulted in the reduction of IFN-γ production by antigen-specific $CD8^+$ T cells. These results suggest that antigens coupled to the surface of liposomes consisting of unsaturated fatty acids might be pinocytosed by APCs, loaded onto the class I MHC processing pathway, and presented to $CD8^+$ T cells. Thus, these liposome-coupled antigens are expected to be applicable for the development of vaccines that induce cellular immunity.

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Introduction

Vaccines have played an important role in disease prevention and have made a substantial contribution to public health. Upon natural infection, it is known that the host responds by inducing both humoral and cellular immunity against the pathogen. However, most of the currently approved vaccines work by inducing humoral immunity [1–3]. For protection against viruses that are highly mutable and frequently escape from antibody-mediated immunity, such as influenza A viruses, HIV, and HCV, humoral immunity is insufficient [4–7]. Consequently, the development of vaccines that induce cellular immunity is critical to novel vaccine strategies.

T lymphocytes respond to peptide fragments of protein antigens that are displayed by MHC molecules on antigen-presenting cells (APCs). In general, extracellular antigens are presented via MHC class II molecules to $CD4^+$ T cells while intracellular antigens are presented via MHC class I molecules to $CD8^+$ T cells [8,9]. However, a number of reports have demonstrated that a significant level of crossover, so-called 'cross-presentation', occurs in APCs [10–14]. Using this phenomenon, novel vaccine preparation inducing antigen-specific CTLs that effectively eliminate virus-infected cells is expected. The mechanisms of cross-presentation have been studied intensively [15–17] while the details have been left unclear. Part of the antigens taken via phagocytosis by APCs are known to be translocated into the cytosol and degraded by local proteases [18,19]. In another pathway, some antigens internalized into endocytic compartments are loaded onto MHC class I molecules [20].

We previously reported that antigens chemically coupled to the surface of liposomes induced antigen-specific IgG but not IgE antibody production [21,22]. In addition, antigens chemically coupled to the surface of liposomes consisting of unsaturated fatty acids were presented not only to $CD4^+$- but also to $CD8^+$ T cells by APCs [23]. Since liposome-coupled antigens induce antiviral immunity [24,25], they are expected to be applicable for the development of viral vaccines without inducing antigen-specific IgEs, which cause allergic reactions. In the present study, we investigated the mechanism by which the liposome-coupled antigens were cross-presented by APCs to $CD8^+$ T cells.

Results

Confocal laser scanning microscopic analysis of macrophages co-cultured with DQ-OVA-liposome conjugates

MHC class I of macrophages were stained with red fluorescein-labeled anti-mouse $H\text{-}2D^d$ mAb (Fig. 1A: left column), and MHC class II of macrophages were labeled with DM-DsRed (Fig. 1A: right column) as described in Materials and Methods. DQ-OVA, which exhibits green fluorescein upon proteolytic degradation, was coupled to liposomes consisting of unsaturated (oleoyl) or saturated (stearoyl) fatty acid, and added to the culture of macrophages. After incubation for 2 hr, the recovered macrophages were analyzed using confocal laser scanning microscopy. The results shown in Fig. 1 demonstrate that DQ-OVA coupled to oleoyl liposomes was processed at both MHC class I and class II compartments, while most of the DQ-OVA coupled to stearoyl liposomes was processed at the MHC class II compartment.

Differential manner of internalization by APCs of antigens coupled to liposomes with two kinds of lipid

$Alexa_{488}$-labeled OVA were coupled to liposomes and were added to the cultures of macrophages. As shown in Fig. 2, OVA coupled to oleoyl liposomes were internalized by APCs more efficiently than those coupled to stearoyl liposomes at 37°C. Interestingly, OVA coupled to oleoyl liposomes but not stearoyl liposomes were internalized significantly by APCs even in a 4°C environment.

Effect of inhibitors on uptake of liposome-coupled antigens by APCs

One of two kinds of inhibitors, cytochalasin B and DMA, which inhibit APC phagocytosis and pinocytosis of antigens, respectively, was added to the culture of macrophages 1 hr prior to the addition of $Alexa_{488}$-OVA- or DQ-OVA-coupled oleoyl liposomes. One hour later, flow cytometric analysis was performed. As shown in Fig. 3, the effect of cytochalasin B on the antigen uptake and digestion of liposome-coupled OVA by APCs was limited. On the other hand, DMA significantly reduced both antigen uptake and digestion of antigens by macrophages.

Localization of antigens coupled to liposomes in APCs

DQ-OVA-coupled oleoyl liposomes were added to the culture of macrophages in which either EEA1 or LAMP-1 were co-stained. The co-localization of the liposome-coupled antigens and intracellular organelles in the APCs was analyzed using confocal laser scanning microscopy. As shown in Figure 4, although most of the DQ-OVA coupled to oleoyl liposomes was processed beyond LAMP-1-expressing compartments (green spots), a portion of DQ-OVA was processed at compartments expressing LAMP-1 (yellow spots). Co-localization of EEA1-expressing compartments with liposome-coupled-DQ-OVA was significantly less than that of LAMP-1-expressing compartments with DQ-OVA (Fig. 4B).

T cell activation by APCs pulsed with liposomal antigen

In agreement with the results shown in Fig. 3, antigen presentation by APCs pulsed with liposomal antigen was significantly inhibited by DMA but not by cytochalasin B in both $CD4^+$- and $CD8^+$ T cell responses (Fig. 5).

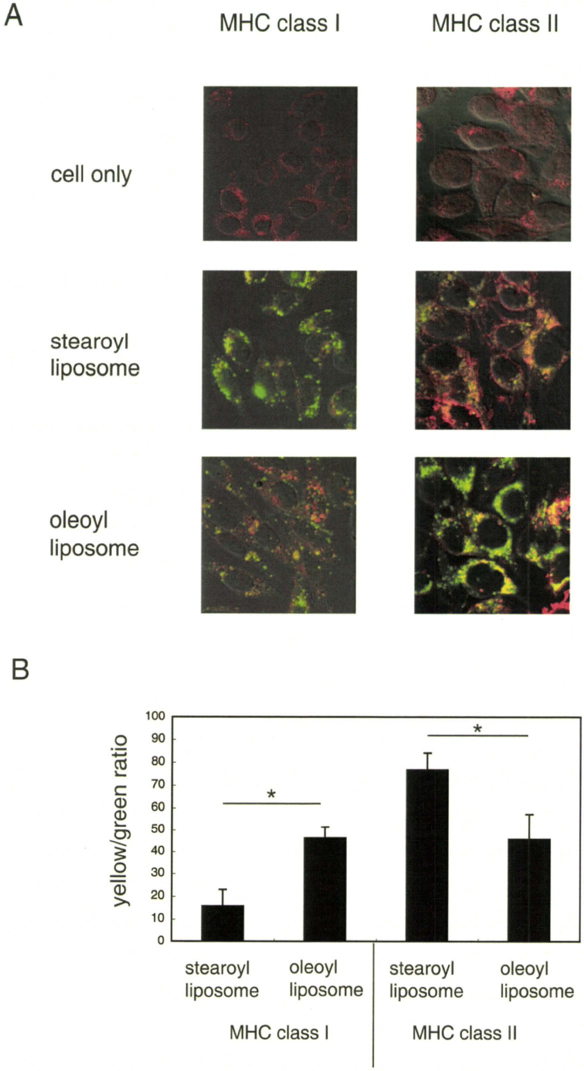
Discussion

In general, extracellular antigens are presented via MHC class II molecules to $CD4^+$ T cells, whereas intracellular antigens are presented via MHC class I molecules to $CD8^+$ T cells. Consequently, most APCs do not present exogenous antigens via MHC class I since exogenous antigens do not gain access to the cytosolic compartment. Therefore, exogenous antigens usually do not prime CTL responses *in vivo*. This segregation of exogenous antigens from the class I pathway is important to prevent CTL from killing healthy cells that have been exposed to foreign antigens but are not infected [26]. However, there are several exceptions to this rule, reflecting the ability of the exogenous antigens to be delivered into the cytosolic compartments [13–17].

We have previously reported that antigens coupled to the surface of liposomes comprised of unsaturated fatty acid are presented to both $CD4^+$- and $CD8^+$ T cells [23]. Confocal laser scanning microscopic analysis demonstrated that a portion of the liposome-coupled antigens were taken up and processed beyond the MHC class II compartment. In the present study, we confirmed that OVA coupled to oleoyl liposomes was processed at both the MHC class I and class II compartments (Fig. 1). Flow cytometric analysis demonstrated that OVA coupled to oleoyl liposomes was incorporated more efficiently by macrophages than OVA coupled to stearoyl liposomes (Fig. 2). Furthermore, OVA coupled to oleoyl liposomes was taken up by macrophages even in a 4°C environment, in which antigen entry could only occur via plasma membrane translocation. In general, antigen processing pathways largely depend on the route of antigen uptake, and liposomes with a certain lipid component are known to fuse with the plasma membrane [27]. The uptake of OVA coupled to oleoyl liposomes in a 4°C environment observed in the present study suggested that oleoyl liposome might fuse with the plasma membrane and thereby allow the liposome-coupled antigen direct access to the cytosol. The role of endocytosis in the uptake of the liposomal antigen was further examined by using specific inhibitors for antigen uptake (Fig. 3). Cytochalasin B treatment of APCs prior to the addition of liposomal antigen in the culture had little effect. However, treatment of APCs with DMA significantly reduced the uptake of liposome-coupled OVA. Consequently, it was suggested that antigens coupled to oleoyl liposomes might be taken up by APCs via at least two pathways, penetration and pinocytosis. The analysis of intracellular pathways of antigens coupled to oleoyl liposomes using confocal laser scanning microscopy demonstrated that a portion of liposomal antigens taken up by APC were translocated to the lysosomal compartments expressing LAMP-1 (Fig. 4), suggesting that the liposomal antigens processed at lysosomal compartment and beyond lysosomal compartment might be presented to CD4/$CD8^+$ T cells via MHC class II and class I, respectively. In agreement with the results of antigen uptake shown in Fig. 3, the treatment of splenic $CD11c^+$ cells with DMA significantly reduced antigen presentation of liposomal antigens to both $CD4^+$- and $CD8^+$ T cells as evaluated by T-cell activation (Fig. 5).

It was reported that pinocytosis and scavenger receptor-mediated endocytosis by APC facilitate antigen presentation to $CD4^+$ T cells; by contrast, mannose receptor-mediated endocytosis by APC has been shown to facilitate antigen presentation to $CD8^+$ T cells [28]. However, as described in Materials and Methods, the oleoyl liposomes used in the present study do not contain mannose.

Thus, the data in the present study demonstrated that antigens coupled to oleoyl liposomes were internalized by APCs through both penetration and pinocytosis. The antigens coupled to the surface of oleoyl liposomes were processed at both MHC class I


A
MHC class I
MHC class II
cell only
stearoyl liposome
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MHC class I
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Figure 1. Confocal laser scanning microscopic analysis of macrophages co-cultured with DQ-OVA-liposome conjugates. A, DQ-OVA was coupled to either stearoyl or oleoyl liposomes and added to the culture of cloned macrophages expressing DM-DsRed (class II) or labeled with red fluorescein (class I), as described in Materials and Methods. Two hours after the onset of the culture, macrophages were recovered and analyzed using confocal laser scanning microscopy. These optically merged images are representative of most cells examined by confocal microscopy. Yellow, co-localization of green (DQ-OVA after proteolytic degradation) and red (macrophage DM or class I); cell only, macrophages without co-culture with DQ-OVA-coupled liposomes. B, the green- and yellow-color compartments in the immunofluorescent pictures were quantified by the image analysis software MetaMorph, as described in Materials and Methods. Ratios of the yellow to green compartments are shown. Data represent the mean values ± SD of the images shown in Fig. 1A. Asterisk, significant ($p<0.01$) difference of samples.
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and class II compartments and presented to $CD4^+$- and $CD8^+$ T cells. Although the detailed pathway leading to presentation to both $CD4^+$- and $CD8^+$ T cells remains unclear, the observed behavior of antigens coupled to oleoyl liposome in APCs seems quite unique. Taken together, coupling of antigens to oleoyl liposome might potentially serve as a novel method to induce both humoral and cellular immunity.

Materials and Methods

Mice

CBF1 mice (8 weeks of age, female) were purchased from SLC (Shizuoka, Japan). All experiments were approved (No. 208021 and 209082) by an independent animal ethics committee at National Institute of Infectious Diseases, Tokyo, Japan.

Chemicals

All phospholipids were provided by NOF Co. (Tokyo, Japan). Reagent grades of cholesterol were purchased from Wako Pure Chemical (Osaka, Japan).

Antigens and reagents

Ovalbumin (OVA, Grade VII) was purchased from Sigma-Aldrich. For the analysis of the processing of liposome-coupled OVA by macrophages, DQ-OVA, which exhibits green fluores-

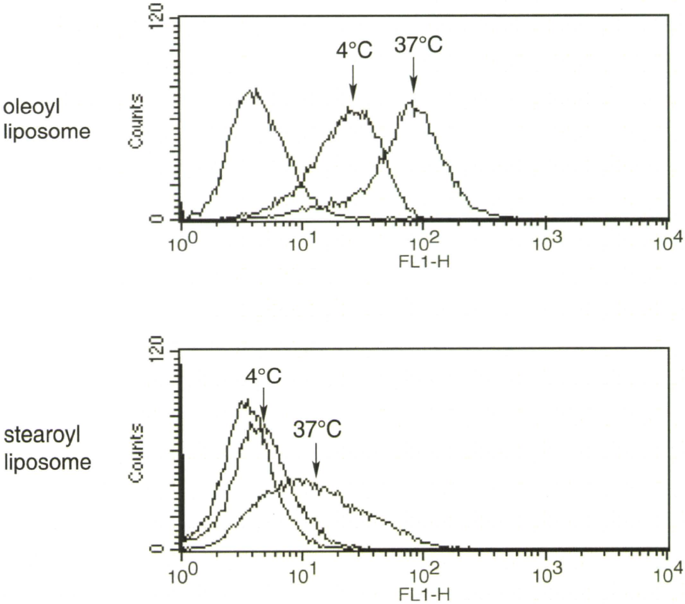

Figure 2. Uptake of liposome-coupled OVA by macrophages. Alexa-labeled OVA was coupled to either stearoyl or oleoyl liposomes and added to the culture of cloned macrophages as described in Materials and Methods. Thirty minutes after the onset of the culture, macrophages were recovered and analyzed using flow cytometry.
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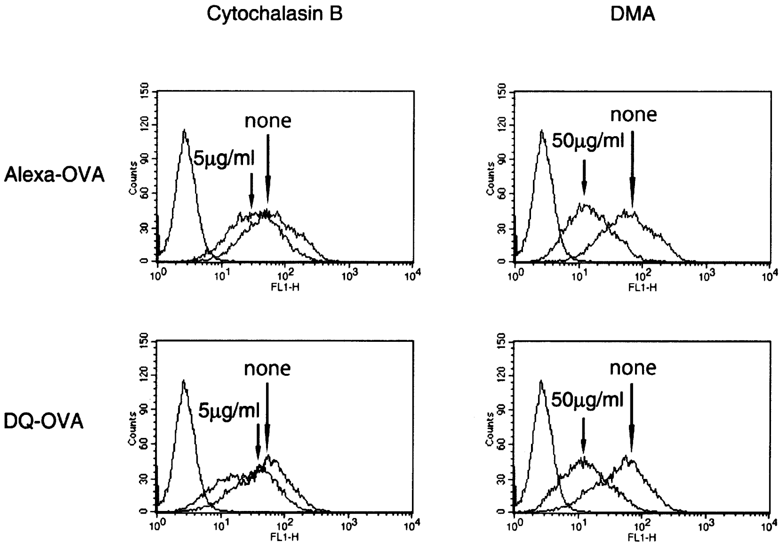

Figure 3. Influence of inhibitors for uptake of OVA coupled to oleoyl liposomes by macrophages. Alexa- or DQ-labeled OVA was coupled to oleoyl liposomes and added to the culture of macrophages as described in Materials and Methods. Treatment of macrophages with cytochalasin B or DMA was done 60 minutes prior to the addition of OVA-liposome conjugates.
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cence upon proteolytic degradation, was purchased from Molecular Probes, Inc. Synthetic CpG ODN (5002: TCCATGACGTTCTTGATGTT) was purchased from Invitrogen and was phosphorothioate-protected to avoid nuclease-dependent degradation.

Fluorescence labeling of OVA

OVA was labeled with fluorescence using an AlexaFluor 488 protein labeling kit (Invitrogen) according to the manufacturer's protocol.

Liposomes

Liposomes consisting of two different kinds of lipid were used in this study. Liposomes consisting of saturated fatty acids were composed of distearoyl phosphatidylcholine, distearoyl phosphatidyl ethanolamine, distearoyl phosphatidyl glycerol acid, and cholesterol in a 4:3:2:7 molar ratio (stearoyl liposomes), and liposomes consisting of unsaturated fatty acids were composed of dioleoyl phosphatidylcholine, dioleoyl phosphatidyl ethanolamine, dioleoyl phosphatidyl glycerol acid, and cholesterol in a 4:3:2:7 molar ratio (oleoyl liposomes). The crude liposome solution was passed through a membrane filter (nucleopore polycarbonate filter, Coster) with a pore size of 0.2 mm.

Coupling of OVA to liposomes

Liposomal conjugates with plain OVA, Alexa-labeled OVA, or DQ-OVA were prepared essentially in the same way as described previously [22]. Briefly, to a mixture of 90 mg of liposomes and 6 mg of OVA in 2.5 ml phosphate buffer (pH 7.2), 0.5 ml of 2.5% glutaraldehyde solution was added in dropwise fashion. The mixture was stirred gently for 1 h at 37°C, and then 0.5 ml of 3 M glycine-NaOH (pH 7.2) was added to block excess aldehyde groups. This was followed by incubation overnight at 4°C. The liposome-coupled OVA and uncoupled OVA in the resulting solution were separated using CL-4B column chromatography (Pharmacia). The amount of lipid in the liposomal fraction was measured using a phospholipid content assay kit (Wako Pure Chemical). The OVA-liposome solution was adjusted to 10 mg lipid/ml in PBS, sterile-filtered using a Millex-HA syringe filter unit (0.45 µm, Millipore), and kept at 4°C until use.

Quantification of OVA coupled to liposome

For the measurement of OVA coupled to liposome, radiolabeled OVA (*methyl*-^{14}C; purchased from New England Nuclear) was mixed with cold OVA and used for coupling with liposome and for determining the calibration curve. The

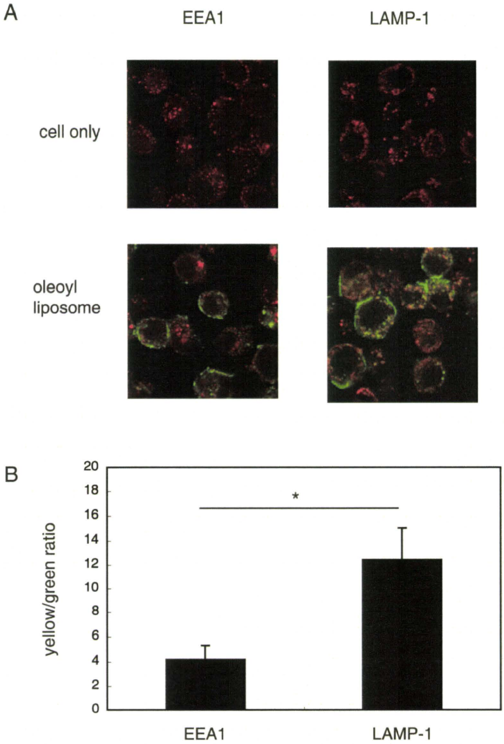

Figure 4. Intracellular localization of liposomal antigens taken up by macrophages. A, DQ-OVA was coupled to oleoyl liposomes and added to the culture of cloned macrophages of which endosomal marker EEA1-positive compartments, or lysosomal marker LAMP-1-positive compartments were stained as described in Materials and Methods. Two hours after the onset of the culture, macrophages were recovered and analyzed using confocal laser scanning microscopy. These optically merged images are representative of most cells examined by confocal microscopy. Yellow, co-localization of green (DQ-OVA after proteolytic degradation) and red (macrophage EEA1 or LAMP-1); cell only, macrophages without co-culture with DQ-OVA liposomes. B, the green- and yellow-color compartments in the immunofluorescent pictures were quantified by the image analysis software MetaMorph, as described in Materials and Methods. Ratios of the yellow to green compartments are shown. Data represent the mean values ± SD of the images shown in Fig, 4A. Asterisk, significant ($p<0.01$) difference of samples.
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radioactivity of the resulting OVA-liposome solution was counted using a calibration curve. The amounts of OVA coupled to stearoyl and oleoyl liposomes were 47.0 and 46.8 μg/mg lipid, respectively.

Immunization

Mice were immunized subcutaneously (s.c.) with the OVA-liposome conjugate at a dose of 1 mg lipid/100 μl/mouse in the presence of 5 μg/mouse CpG.

Cloned macrophage hybridoma

Macrophage hybridoma clone 39, obtained from the fusion of splenic adherent cells from CKB mice and P388D1 [29], was used.

Construction and expression of the fusion protein, DM-DsRed, in macrophage clone 39

The DNA fragment coding the full-length H2-DMβ2 [30] was amplified by PCR with two primers (5′-ATGGCTGCACT-CTGGCTGCTGCTGCTGGT-3′ and 5′-GATGCCGTCCT-

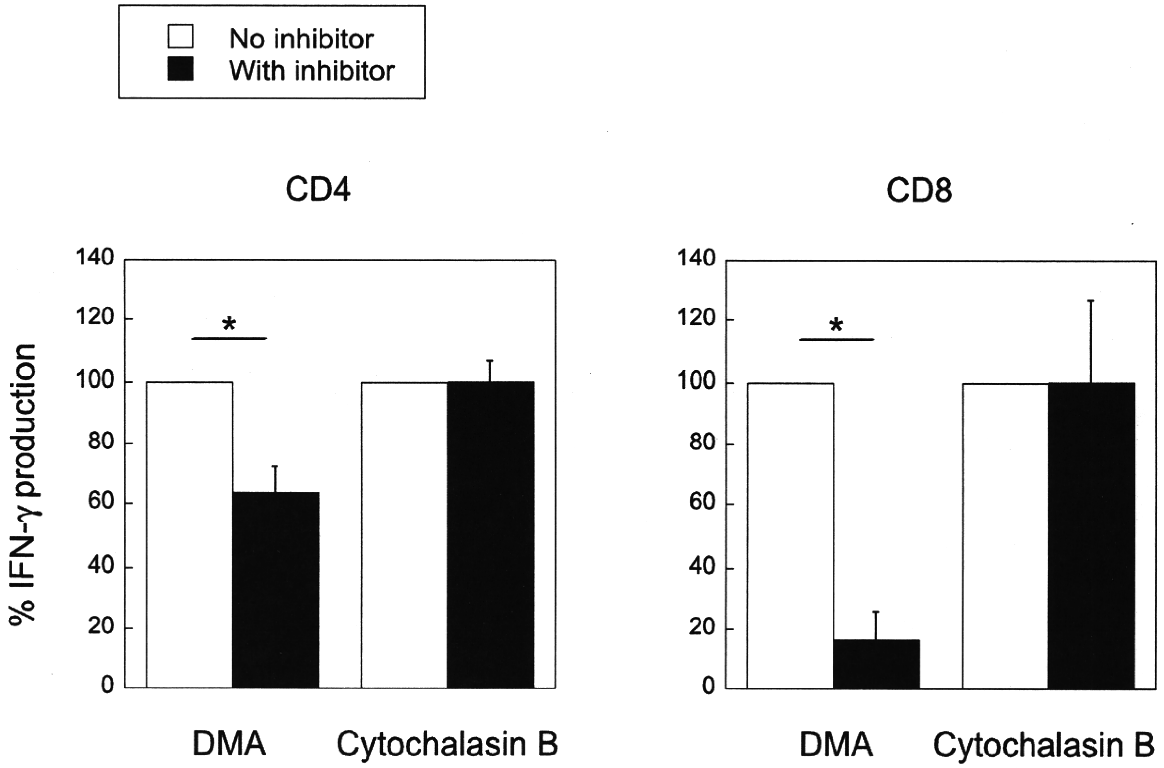

Figure 5. IFN-γ production by splenic CD4/CD8$^+$ T cells of mice immunized with OVA after co-culture with CD11c$^+$ cells pulsed with OVA coupled to oleoyl liposomes. Splenic CD4/CD8$^+$ T cells were taken from mice immunized with OVA and were cultured with CD11c$^+$ cells pulsed with OVA coupled to oleoyl liposomes with or without inhibitors as described in Materials and Methods. IFN-γ production of T cells in the supernatants in the absence of inhibitors was normalized to 100%. Data represent the mean values ± SD of triplicate culture. Asterisk, significant ($p<0.01$) difference as compared with the 'no inhibitor' group.
doi:10.1371/journal.pone.0015225.g005

TCTGGGTAGGTGGATCC-3′). The PCR product was cloned into the CMV promoter-driven expression plasmid pDsRedN1 (BD Clontech). This construct omitted the stop codon of H2-DMβ2 and encoded the H2-DMβ2 fused with DsRed. The cloned plasmid DNA was transfected to macrophage hybridoma clone 39 with Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. During the transfection to clone 39, the medium containing cDNA and the transfection reagent was replaced with fresh medium after an 8-h transfection, and then clone 39 was cultured for 40 h. To obtain stable cell lines, clone 39 was passaged at 1:5 into RPMI 1640 containing 10% FCS with 50 μg/ml geneticin (G-418; Sigma-Aldrich). Cells showing the best fluorescence were selected using a FACS Vantage cell sorter (BD Bioscience). After cell sorting, clone 39 expressing DM-DsRed was cultured in RPMI 1640 containing 10% FCS with 200 μg/ml geneticin.

Flow cytometry

To investigate the capture of OVA-liposome conjugates by macrophages, macrophage clone 39 was incubated for 30 min at 4°C or 37°C in the presence of fluorescence-labeled OVA-liposome conjugates that contained a final concentration of 4 μg/ml OVA. After the incubation, cells were washed with ice-cold PBS. In the case of using Alexa-labeled OVA-liposome conjugates, cells were then incubated with 1.2 μg/ml trypan-blue for 5 min at 4°C to block the fluorescence of Alexa-OVA attached to the cell surface. After the cells were washed, they were analyzed on a FACS Caliber flow cytometer (BD Bioscience). The histograms of fluorescence distribution were plotted as the number of cells versus fluorescence intensity on a logarithmic scale.

Confocal laser scanning microscopy

To investigate the localization of OVA-liposome conjugates by macrophages, macrophage clone 39 or DM-DsRed-expressing cloned macrophage 39 was cultured for 18 h at 37°C on 8-hole heavy Teflon-coated slides (Bokusui Brown) and was then incubated with DQ-OVA-liposome conjugates, prepared using oleoyl or stearoyl liposomes, for 2 h at 37°C. The slides were then washed with MEM and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After fixation, they were incubated for 10 min in 0.1 M glycine-HCl (pH 7.0) to block the remaining aldehyde residue. They were then washed two times in PBS. After washing, the slides were sealed with PBS:glycerin (1:9) and analyzed under an LSM510 confocal laser scanning microscope system (Zeiss). For analysis of co-localization of OVA and MHC class I, early endosomal antigen 1 (EEA1) or lysosomal-associated membrane protein-1 (LAMP-1) after blocking of the remaining aldehyde residue, cloned macrophage 39 was subsequently permeabilized with 0.05% saponin-TBS for 10 min at room temperature. After being washed twice with PBS, they were reacted with biotin-conjugated mouse anti-mouse H-2D^d mAb (34-2-12, 10 μg/ml; BD Biosciences), goat anti-mouse EEA1 polyclonal antibody (N19, 1 μg/ml; Santa Cruz Biotechnology) or rat anti-mouse LAMP-1 monoclonal antibody (1D4B, 1 μg/ml; Santa Cruz Biotechnology) for 18 h at 4°C. After being washed three times with TBS, they were reacted with Alexa 546-conjugated streptavidin (1:200 diluted; Invitorogen) to detect MHC class I, Alexa Fluor 568-labeled Ab (rabbit anti-goat IgG, 10 μg/ml; Invitrogen) to detect EEA1 or Alexa Fluor 568-labeled Ab (goat anti-rat IgG, 10 μg/ml; Invitrogen) to detect LAMP-1 for 4 h at room temperature. They were then washed two times in TBS. After the washing, the slides were sealed with PBS:glycerin (1:9) and analyzed under an LSM510 confocal laser scanning microscope system (Zeiss).

Quantification of immunofluorescent pictures and statistics

Quantification of confocal image analysis was done by single cell identification using the image analysis software MetaMorph (Molecular Devices Co., Tokyo, Japan), and the relative fluorescence intensity of green, red, and yellow pixels was assessed. The relative fluorescence intensity of all individual colors was then expressed as percent of the total fluorescence intensity. *p* values were calculated by the Student's t test with two-tailed distribution and two-sample unequal variance parameters.

Inhibition Studies of Antigen Uptake

In the case of inhibition studies, cloned macrophage 39 or CD11c$^+$ cells were incubated with indicated inhibitors 60 min before and throughout the antigen pulse. Cytochalasin B [31] and DMA [28] were purchased from Sigma.

Preparation of CD11c$^+$ cells and CD4$^+$- and CD8$^+$ T cells

CD11c$^+$ spleen cells of naïve mice and CD4$^+$ T and CD8$^+$ T spleen cells of mice immunized with OVA-liposome conjugates were prepared with the magnetic cell sorter system MACS, according to the manufacturer's protocol using anti-CD11c, anti-CD4 and anti-CD8 antibody-coated microbeads (Miltenyi Biotec).

Culture of CD4$^+$- and CD8$^+$ T cells with CD11c$^+$ cells pulsed with OVA

CD11c$^+$ cells were incubated with or without the indicated inhibitors for 60 min in a 24-well plate prior to the addition of OVA-liposome conjugates made using oleoyl liposomes. The final concentration of OVA-liposome added to the macrophage culture was 500 μg lipid/ml, which included 24 μg OVA. After 60 minutes' incubation, CD11c$^+$ cells were washed 3 times in ice-cold medium and 2×10^5 cells were co-cultured with 5×10^5 CD4$^+$ T cells or CD8$^+$ T cells, in a 48-well plate. A preliminary experiment showed that the optimal culture period in the above culture condition was 2 days for IFN-γ production by CD4$^+$ T cells and 5 days for IFN-γ production by CD8$^+$ T cells. After incubation in a CO_2 incubator for 2 or 5 days, the culture supernatants were collected and assayed for IFN-γ.

IFN-γ Assay

IFN-γ in the culture supernatants was measured using the Biotrak mouse ELISA system (GE Healthcare). All test samples were assayed in duplicate, and the SD in each test was always <5% of the mean value.

Author Contributions

Conceived and designed the experiments: MT TU. Performed the experiments: YT MT TK TU. Analyzed the data: MT MK TU. Contributed reagents/materials/analysis tools: MT TU. Wrote the paper: MT TU.

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Short communication

Detection of all known filovirus species by reverse transcription-polymerase chain reaction using a primer set specific for the viral nucleoprotein gene

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ABSTRACT

The filoviruses, Marburg virus (MARV) and Ebola virus (EBOV), are causative agents of severe hemorrhagic fever with high mortality rates in humans and non-human primates. Sporadic outbreaks of filovirus infection have occurred in Central Africa and parts of Asia. Identification of the natural reservoir animals that are unknown yet and epidemiological investigations are current challenges to forestall outbreaks of filovirus diseases. The filovirus species identified currently include one in the MARV group and five in the EBOV group, with large genetic variations found among the species. Therefore, it has been difficult to develop a single sensitive assay to detect all filovirus species, which would advance laboratory diagnosis greatly in endemic areas. In this study, a highly sensitive universal RT-PCR assay targeting the nucleoprotein (NP) gene of filoviruses was developed. The genomic RNAs of all known MARV and EBOV species were detected by using an NP-specific primer set. In addition, this RT-PCR procedure was verified further for its application to detect viral RNAs in tissue samples of animals infected experimentally and blood specimens of infected patients. This assay will be a useful method for diagnostics and epidemiological studies of filovirus infections.

Marburg virus (MARV) and Ebola virus (EBOV) are enveloped, single-stranded, negative-sense RNA viruses classified into two genera, *Marburgvirus* and *Ebolavirus*, in the family *Filoviridae*, order *Mononegavirales* (Feldmann et al., 2004). These viruses are causative agents of severe hemorrhagic fever with high mortality rates in humans and non-human primates (Feldmann et al., 2003). There is a single *Marburgvirus* species, *Lake Victoria marburgvirus* (LVMARV), whereas there are four known *Ebolavirus* species, *Zaire ebolavirus* (ZEBOV), *Sudan ebolavirus* (SEBOV), *Reston ebolavirus* (REBOV) and *Cote d'Ivoire ebolavirus* (CIEBOV) (Feldmann et al., 2004). The genomic structures of filoviruses are very similar and approximately 19 kilobases in length, containing seven genes arranged sequentially in the order nucleoprotein (NP)-viral protein (VP) 35-VP40-glycoprotein-VP30-VP24-RNA polymerase (L) (Sanchez et al., 2006).

Since the discovery of Marburg hemorrhagic fever in Germany in 1967, sporadic outbreaks of Marburg and Ebola hemorrhagic fever have been reported from different countries in Central Africa (Feldmann et al., 2003). Incidences have increased in Central Africa since the beginning of the new millennium (Centers for Disease Control and Prevention, 2010a, b), and *Bundibugyo ebolavirus* (BEBOV) which has been proposed as a fifth species of EBOV was discovered recently in Uganda (Towner et al., 2008). Furthermore, two imported cases of Marburg hemorrhagic fever (in the Netherlands and the United States) (Centers for Disease Control and Prevention, 2009; Timen et al., 2009) and one of Ebola hemorrhagic fever (in South Africa) (World Health Organization, 1997) in travelers, have been reported, emphasizing the risk of filovirus infection in non-endemic countries.

Real-time RT-PCR (Drosten et al., 2002; Gibb et al., 2001; Weidmann et al., 2004) and reverse transcription-loop-mediated isothermal amplification (RT-LAMP) methods (Kurosaki et al., 2007, 2010) have been published recently for the diagnosis of filovirus infections. However, the real-time RT-PCR requires expensive, sophisticated equipment and thus does not seem to be practical for routine use in endemic areas such as Africa, and false-positive reactions in RT-LAMP cannot be discriminated since it does not

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provide valuable nucleotide sequence information on its products. More importantly, these methods established previously are relatively species-specific and cannot be applied reliably in diagnostics and field studies for the broad detection of filoviruses, including potential new species. For example, this was the case for BEBOV infections, which were not detected initially by using real-time RT-PCR specific for the filoviruses species known previously, ZEBOV, SEBOV, and MARV (Towner et al., 2008). In fact, only one sample out of 20 human blood specimens suspected was positive in conventional RT-PCR using the currently available universal filovirus primers, FILO-A and FILO-B (Sanchez et al., 1999; Towner et al., 2008). Therefore, it is important to design new primers for RT-PCR allowing the broad detection of filovirus genomic RNA. In this study, nucleotide sequences of all known filovirus species were compared and a new assay for the detection of all known filoviruses using a primer set targeting a highly conserved region in the NP gene was evaluated.

One-step RT-PCR was carried out using a QIAGEN OneStep RT-PCR Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. A total volume of 25 μl of reaction mixture containing 0.2 μM of each primer and 1 μl of template RNA was used. Four primers targeting MARV and EBOV NP genes (FiloNP primers) were designed (Table 1). The one-step RT-PCR program consisted of reverse transcription at 50 °C for 30 min, initial PCR activation at 95 °C for 15 min, followed by 50 cycles of denaturation at 94 °C for 15 s, annealing at 53 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 7 min (Veriti 200 thermal cycler; Life Technologies Co., Carlsbad, CA). For comparison, the primers published previously, FILO-A and FILO-B, were used under the same conditions.

LVMARV (Popp, Ozolin, Musoke, Ravn, and Angola #368), ZEBOV (Mayinga and Kikwit), SEBOV (Boniface), CIEBOV (Cote d'Ivoire), BEBOV, and REBOV (Pennsylvania) strains were used in this study. These viruses were propagated in Vero E6 cells, and viral RNAs were extracted from 250 μl aliquots of culture supernatants using TRIzol-LS reagent (Invitrogen Co., Carlsbad, CA) according to the manufacturer's instructions. The extracted RNA was dissolved in 20 μl of nuclease-free distilled water. All infectious materials were handled in the biosafety level 4 facility of the National Microbiology Laboratory, Public Health Agency of Canada. Lassa virus, hantavirus, dengue 2 virus, and leptospira interrogans RNAs were used as specificity controls because of the similarity of disease symptoms and/or potential endemic area shared with filoviruses.

As shown in Fig. 1A, NP gene fragments of 5 MARV strains and 6 EBOV strains were amplified similarly as 594 bp products by the NP primer combination designed in this study. Nucleotide sequences of all the products were determined and identified as those derived from the respective template strains (data not shown). There was no nonspecific amplification of the Lassa virus and hantavirus RNAs tested. Furthermore, assays using FiloNP-Fm and FiloNP-Rm or FiloNP-Fe and FiloNP-Re primer combinations amplified separately MARV and EBOV RNAs, respectively (Fig. 1B), demonstrating the usefulness of these primer sets in differentiating between MARV and EBOV strains. In contrast, the primers published previously, FILO-A and FILO-B, failed to detect LVMARV Ravn, CIEBOV Cote d'Ivoire, and REBOV Pennsylvania strains (Fig. 1A). The lower stability of the primer match of FILO-A and FILO-B than NP primers, as seen in the alignments between primers and virus genomes, likely influenced the efficiency of amplification (Fig. 2).

To determine the sensitivity of the RT-PCR assay using NP primers, viral RNAs derived from supernatants of samples infected LVMARV strain Angola #368 (approximately 10^7 plaque-forming units (PFU)/ml) (Geisbert et al., 2007), and ZEBOV strain Mayinga (approximately 10^7 focus forming units (FFU)/ml), were diluted serially 10-fold in nuclease-free distilled water, and used as templates for amplification. The detection limits for LVMARV strain Angola #368, and ZEBOV strain Mayinga were approximately 10^{-2} to 10^{-1} PFU or FFU/reaction (Fig. 1C) and thus equivalent to the reported sensitivity for the universal primers designed previously (i.e. FILO-A and FILO-B). Although the RT-PCR may not be more sensitive than the TaqMan RT-PCR (Weidmann et al., 2004) and RT-LAMP (Kurosaki et al., 2007, 2010) established previously, the simplicity and cross-reactivity among all known filovirus strains provides an advantage for rapid diagnostics in reference centers and field settings.

Finally, the applicability of RT-PCR using NP primers to *in vivo* diagnostics was studied. Total RNA was extracted from 100 μl of a 10% (w/v) spleen homogenate derived from mice infected with mouse-adapted ZEBOV (titer approximately 10^7 FFU/g) (Ebihara et al., 2006) by using TRIzol-LS reagent (Invitrogen Co.) according to the manufacturer's instructions. The extracted RNA, dissolved in 30 μl of nuclease-free distilled water, was diluted serially 10-fold in nuclease-free distilled water and used as a template. Viral gene fragments were amplified successfully with the detection limit of approximately 10^{-3} FFU/reaction (Fig. 3A), demonstrating that the sensitivity of the RT-PCR assay using NP primers was equivalent to that of the RT-PCR using FILO-A and FILO-B reported previously (Sanchez et al., 1999). Subsequently, whole-blood samples from Marburg hemorrhagic fever cases in Angola in 2004/05 (World Health Organization, 2005) were analyzed. Total RNA was extracted from 100 μl of patient blood samples and dissolved in 10 μl of

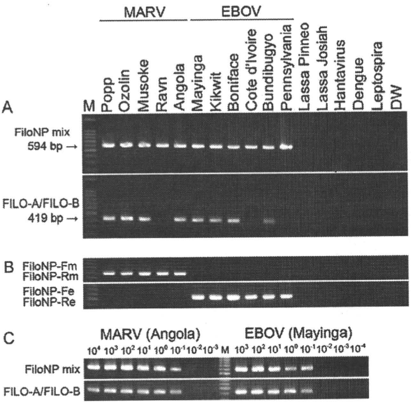

Fig. 1. Specificity and sensitivity of RT-PCR for the detection of MARV and EBOV. (A) Filovirus NP gene fragments were amplified by RT-PCR using a mixture of 4 primers, FiloNP-Fm, FiloNP-Rm, FiloNP-Fe, and FiloNP-Re (upper panel), or FILO-A and FILO-B (lower panel). Lassa virus (strains Pinneo and Josiah), hantavirus (species Dobraba, strain Slovenia), dengue 2 virus (strain VNHCM18-C/02), and leptospira interrogans (serovar Manilae, strain UP-MMC-NIID) RNAs were used to verify the specificity of the RT-PCR. Amplification of these control RNAs by RT-PCR was confirmed by using specific primers for the respective pathogens (data not shown). Nuclease-free distilled water (DW) was used as a negative control. Data are representative of two independent experiments. (B) MARV and EBOV NP genes were detected by FiloNP-Fm and FiloNP-Rm (upper panel) and FiloNP-Fe and FiloNP-Re (lower panel) primer sets, respectively. Lassa virus and hantavirus RNAs were used to verify the specificity of the RT-PCR. Data are representative of two independent experiments. (C) Tenfold serial dilutions of RNA derived from LVMARV, strain Angola (left) and ZEBOV, strain Mayinga (right) were analyzed; approximate virus titers (PFU for strain Angola and FFU for strain Mayinga) are shown at the top of the panel. Primers FiloNP-Fm, FiloNP-Rm, FiloNP-Fe, and FiloNP-Re (upper panel) and FILO-A and FILO-B (lower panel) were used for amplification. Data are representative of three independent experiments. Lane M: 100-bp DNA ladder.

Table 1
Primers used in this study.

Primer	Sequence (5′–3′)	Target gene (position)	Product size	Reference strain (accession no. [a])	Reference
FiloNP-Fm	TGGCTTACYACAGGYCACATGAAAGT	MARV NP (620–645)	594 bp	MARV Musoke (NC_001608)	This study
FiloNP-Rm	GTGTGTGATTTCAGTTTTYTGGAGGTGGAA	MARV NP (1213–1184)			
FiloNP-Fe	TGGCAATCAGTDGGACACATGATGGT	EBOV NP (1040–1065)	594 bp	EBOV Mayinga (NC_002549)	This study
FiloNP-Re	GAAGCTGATTTCRTTCTTYTTCTGATGGAA	EBOV NP (1633–1604)			
FILO-A	ATCGGAATTTTTCTTTCTCATT	Filovirus L (13123–13144)	419 bp	MARV Musoke (NC_001608)	Sanchez et al. (1999)
FILO-B	ATGTGGTGGGTTATAATAATCACTGACATG	Filovirus L (13541–13512)			

[a] Accession no. indicates Genbank accession number of reference sequence.

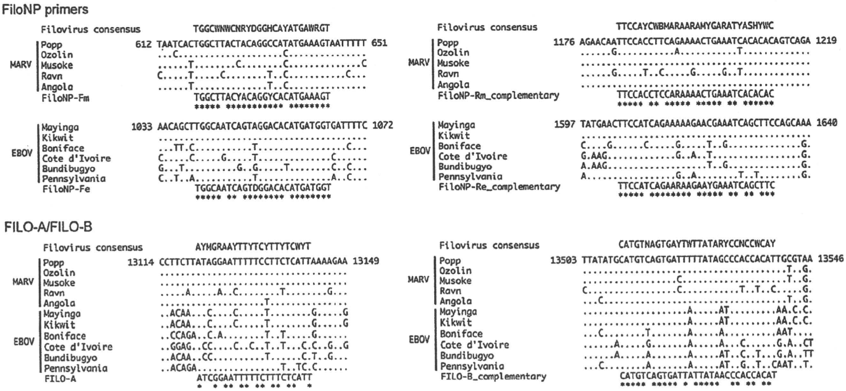

Fig. 2. Alignment of filovirus NP and L gene sequences and primer sequences used in this study. Reverse primers are shown as complementary sequences. Dots indicate the positions identical to LVMARV strain Popp, or ZEBOV strain Mayinga sequences. The numbers on the left and right indicate the respective nucleotide positions in the Popp and Mayinga strain genome sequences. Asterisks indicate the positions matching the primer sequences. Genbank accession numbers of the nucleotide sequences used in this study are Z29337 (Popp), AY358025 (Ozolin), NC_001608 (Musoke), EF446131 (Ravn), DQ447656 (Angola), NC_002549 (Mayinga), AY354458 (Kikwit), FJ968794 (Boniface), FJ217162 (Cote d'Ivoire), FJ217161 (Bundibugyo), and AY769362 (Pennsylvania).

nuclease-free distilled water. Specific amplification of MARV NP gene sequences by the NP primer set was confirmed in all the samples tested, as with the primer set of FILO-A and FILO-B reported previously (Fig. 3B), demonstrating the applicability of this assay to human diagnostics.

In this study, NP-gene-specific primers were designed. NP transcripts were detected *in vitro* as early as 7 h after infection and the number of NP transcript copies was abundant, in contrast to the much lower copy numbers of RNA transcripts derived from the L gene (Sanchez and Kiley, 1987). Thus, this RT-PCR assay using primer sets targeting the NP gene is expected to be more sensitive than those based on L-gene-specific primers to detect cell-associated filovirus RNA transcripts in infected primary target cells such as peripheral monocytes in the early stage of infection. The

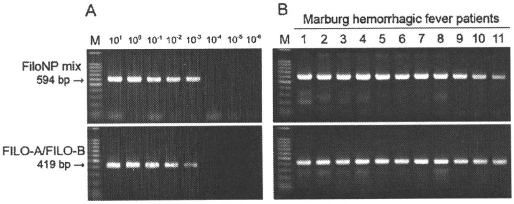

Fig. 3. Detection of EBOV and MARV in experimental animal and human specimens. The NP gene fragments were amplified by RT-PCR using a combination of 4 primers, FiloNP-Fm, FiloNP-Rm, FiloNP-Fe, and FiloNP-Re (upper panels), or FILO-A and FILO-B (lower panels). (A) Tenfold serial dilutions of RNA extracted from ZEBOV-infected mouse spleen were used as templates; approximate virus titers (FFU) used in each reaction are shown at the top of the panel. Data are representative of three independent experiments. (B) MARV-infected human blood specimens obtained during the 2004/05 Marburg hemorrhagic fever outbreak in Angola were analyzed for the presence of MARV RNA. Blood collection from humans during the outbreak in Angola was approved under a special response protocol established between the World Health Organization and national authorities. Lane M: 100-bp DNA ladder.

broad cross-reactivity of RT-PCR with the NP primers designed in this study compared to the ones reported previously will enhance filovirus PCR diagnostics and thus provide a novel tool for public health and biodefense. This combined with its simplicity will also improve ecological and epidemiological field studies in regions with poor infrastructure in Central Africa.

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