Development of a novel T cell-oriented vaccine using CTL/Th-hybrid epitope long peptide and biodegradable microparticles, against an intracellular bacterium

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Abbreviation list

BCG: Bacille Calmette-Guerin

BMDC: bone marrow-derived dendritic cell

CFSE: carboxyfluorescein diacetate succinimidyl ester

CFU: colony forming units

CTL: cytotoxic T-lymphocytes

DC: dendritic cell

IFN-γ: interferon-γ

LLO: listeriolysin O

LPS: lipopolysaccharide

OVA: ovalbumin

PLGA: poly (lactic-co-glycolic acid)

TCR: T-cell receptor

Th: helper T-lymphocytes

Abstract

Antigen-specific CD8+ T-lymphocytes (cytotoxic T-lymphocytes: CTL), as well as CD4+ T-lymphocytes (helper T-lymphocytes: Th), simultaneously play an important role in elimination of intracellular bacteria such as *Mycobacterium tuberculosis* and *Listeria monocytogenes*. Administration of T-cell epitope short peptide needs large numbers of peptides for effective vaccination due to its easily degradable nature *in vivo*. In this respect, biocompatible and biodegradable microparticles combined with CTL/Th-hybrid epitope long peptide (long peptide) have been used to diminish the loaded peptide degradation. The aim of this study is to develop a novel T cell-oriented vaccine against intracellular bacteria that is composed of long peptide and poly (lactic-co-glycolic acid) (PLGA) microparticles. Mouse bone marrow-derived dendritic cells (BMDCs) were loaded with *L. monocytogenes* listeriolysin O (LLO)-derived or ovalbumin (OVA)-derived long peptide/PLGA or other comparative antigens. The antigen-loaded BMDCs were subcutaneously injected into flank of mice twice, and then, the spleens were collected and lymphocyte proliferation and interferon-γ production were evaluated. The median diameter of PLGA spheres was 1.38 μm. Both LLO- and OVA-long peptide/PLGA showed significantly more robust CTL and Th proliferations with higher interferon-γ production than the long peptide alone or CTL and Th short peptides/PLGA vaccination. Furthermore, the LLO-long peptide/PLGA vaccination showed significantly lower bacterial burden in spleens compared with the long peptide alone or the CTL and Th short peptides/PLGA vaccination after the challenge of lethal amounts of *L. monocytogenes*. These results suggest that the novel vaccine taking advantages of CTL/Th-hybrid epitope long peptide and PLGA microparticle is effective for protection against intracellular bacteria.

Keywords: CTL/Th-hybrid epitope long peptide; Cross-presentation; Intracellular bacteria; Microparticle; PLGA

1. INTRODUCTION

Intracellular bacterial infection, especially by *Mycobacterium tuberculosis*, is a significant cause of death worldwide [1]. An estimated 10 million people develop tuberculosis and 1.3 million people are estimated to die from this infection each year [1]. Although Bacille Calmette-Guerin (BCG) vaccine can protect children from severe tuberculosis, this protective capacity is not enough or cannot induce long-term immunity in the adult population [1]. Because of the low safety of live attenuated vaccines in immunocompromised individuals and their variable effectiveness, the development of new, improved vaccines against intracellular bacteria is a current research priority.

Host cellular immune response is extremely important for protection against intracellular bacteria. Antigen-specific CD4+ T-lymphocytes (helper T-lymphocytes: Th), especially interferon-γ (IFN-γ) producing Th1 cells, play an important role in elimination of intracellular bacteria [2]. CD8+ T-lymphocytes (cytotoxic T-lymphocytes: CTL) are also significant for elimination of cells infected by intracellular bacteria [3]. CD4+ T-cell help facilitates interaction between dendritic cells (DCs) and CTL, and downregulates PD-1 expression, consequently enhancing CTL response [4]. In addition, to induce an antigen-specific CTL response, antigen cross-presentation in DC is indispensable [5, 6], and CD4+ T-cell help also enhances this cross-presentation [7].

Listeria monocytogenes is a Gram-positive facultative intracellular bacterium that causes life-threatening infection during pregnancy and in immunocompromised individuals. Murine *L. monocytogenes* infection has been studied as a good model system for intracellular bacterial infection to evaluate the protective immune system [8]. We reported that single CTL or Th epitope DNA vaccination induces protective immunity [9, 10]. Listeriolysin O (LLO) is the critical virulence factor and also a T-cell target molecule of *L. monocytogenes*. $LLO_{91\text{-}99}$ was reported as an immunodominant CTL epitope in BALB/c mice ($H2^d$) [11]. $LLO_{215\text{-}226}$ was first

identified as a Th epitope in C3H mice ($H2^k$), and we previously confirmed that $LLO_{215-226}$ worked as an immunodominant Th1 epitope also in BALB/c mice by DNA vaccination [12].

CTL-epitope peptide is composed of 8–10 amino acids crucial for antigen-specific CTL induction. However, the peptide itself is easily degradable *in vivo,* and vaccination with it needs a large amount of the peptide and is highly expensive. In addition, administration of CTL-epitope peptide induces immunologic tolerance via interaction with T- and B-cells but not with DCs [13]. In contrast, synthetic epitope long peptide is more stable than short peptide, and can avoid immunologic tolerance [13]. Furthermore, T-cell epitope long peptide facilitates DC cross-presentation [14]. Takahashi et al. [15] reported artificially synthesized T-cell epitope long peptide comprising CTL-epitope peptide and Th-epitope peptide with a glycine linker, and administration of the peptide could decrease pulmonary metastasis of colon cancer. Therefore, CTL/Th-hybrid epitope long peptide vaccination is a promising strategy to simultaneously induce both CTL and Th responses.

Biodegradable micro- or nano-particles have been used as vaccine or drug delivery carriers. One of the most successfully used biomaterials for the particles is poly (lactic-co-glycolic acid) (PLGA). PLGA is safe and clinically approved by the USA Food and Drug Administration. It is reported to diminish the loaded protein or peptide degradation, and extends the antigen-presentation period by antigen-presenting cells (APCs) [16]. Furthermore, PLGA particles also facilitate DC cross-presentation [17, 18]. These results led us to the hypothesis that a novel vaccine in combination with CTL/Th-hybrid epitope long peptide and PLGA particles may be able to facilitate antigen cross-presentation and to simultaneously induce strong CTL and Th responses against intracellular bacteria.

In the present study, we developed the novel vaccine taking advantages of CTL/Th-hybrid epitope long peptide and PLGA particle, and evaluated the efficacy of this vaccine against intracellular bacteria. To the best of our knowledge, this is the first report to show that the

combination of CTL/Th-hybrid epitope long peptide and PLGA particles could induce strong CTL/Th responses and protective immunity against an intracellular bacterial infection.

2. MATERIALS AND METHODS

2.1. Mice

Female C57BL/6 and BALB/c (8–12 weeks of age) mice were purchased from SLC Japan (Hamamatsu, Japan). OT-I and OT-II T-cell receptor (TCR)-transgenic mice were from the Jackson Laboratory (Sacramento, CA) and the Center for Animal Resources and Development in Kumamoto University, respectively. OT-I mice carry a transgenic TCR specific for fragment 257-264 of ovalbumin (OVA) protein in association with H2-K^b [19], while OT-II mice carry a transgenic TCR specific for fragment 323-339 of OVA protein in association with H2-A^b [20]. All experimental protocols were approved by the Animal Care and Use Committee of Hamamatsu University School of Medicine and all experiments were conducted according to guidelines of this Committee.

2.2. *In vitro* culture media and reagents

All cultures were in a complete medium composed of Iscove's modified Dulbecco's medium, 10% heat inactivated fetal bovine serum, and 1% penicillin-streptomycin (all from Invitrogen, Carlsbad, CA). Lipopolysaccharide (LPS) from *Escherichia coli* O111 was from Sigma-Aldrich (St. Louis, MO).

2.3. Culture of bone marrow-derived dendritic cells (BMDCs)

To generate BMDCs, bone marrow cells from C57BL/6 or BALB/c mice were cultured at 4×10^5 cells/ml in complete medium containing 400 U/ml IL-4 and 1000U /ml GM-CSF for 7

days, as described [21], and activated by adding 100 ng/ml LPS during the last 16 hours of culture. BMDCs were collected from the culture by gentle pipetting and were ≥80% CD11c-positive by flow cytometry.

2.4. T-cell epitope short and long peptides

As for T-cell epitope short peptides, the $OVA_{257–264}$ (SIINFEKL) and $OVA_{323-339}$ (ISQAVHAAHAEINEAGR) peptides were from MBL (Nagoya, Japan). LLO_{91-99} (GYKDGNEYI) and $LLO_{215-226}$ (SQLIAKFGTAFK) were from MBL and SCRUM (Tokyo, Japan), respectively. In terms of T-cell epitope long peptides, as shown in FIGURE 1, OVA epitope long peptide was synthesized by conjugating CTL ($OVA_{257-264}$) and Th ($OVA_{323-339}$) epitope peptides with glycine-linker [22] in Fmoc solid-phase peptide synthesis method (SCRUM; FIGURE 1A). In the same way, we synthesized LLO T-cell epitope long peptide by conjugating CTL (LLO_{91-99}) and Th ($LLO_{215-226}$) epitope peptides with glycine-linker (SCRUM; FIGURE 1B). Synthesized peptides were purified by high-pressure liquid chromatography in >95% purity.

2.5. PLGA microparticles

PLGA microparticles were purchased from Phosphorex (Hopkinton, MA). Microparticles were prepared by the water-in-oil-in-water (w/o/w) modified double emulsion solvent extraction technique [23, 24]. Briefly, 0.2 mg of PLGA was dissolved in 1 ml of methylene chloride. Then antigens (epitope short or long peptides) in PBS with 50% acetonitrile, 0.25 mM NaOH, and 50 mM Hepes was added to PLGA solution (w/o: primary emulsion). Then, the w/o emulsion was poured slowly in 10 ml of 2.5% W/V polyvinyl alcohol (w/o/w: secondary emulsion). The secondary emulsion was agitated as fast as possible using a magnetic stirrer at 40°C in the safety hood for 2 hours until the methylene chloride evaporated.

Finally, the microparticles were washed with PBS and lyophilized in a freeze-dryer (Speed Vac® Plus SC110A, Savant instruments, Holbrook, NY) for 6 hours, and stored at -30°C for later use. Scanning electron microscopy (S-4800, HITACHI, Tokyo, Japan) was used to confirm size distribution and morphology of the particles. The sizes of the particles were determined using Image-J (US NIH, Bethesda, MD).

To evaluate the release profile of peptide ($OVA_{257-264}$ and LLO_{91-99}) from microparticles, 0.2 mg of PLGA particles was resuspended in 1 ml of PBS. Tubes were kept under agitation at 100 rpm at 37°C. At each time point, microparticles were centrifuged and supernatant were collected and stored at -30°C for further analysis. Then, PLGA particles were replenished with fresh PBS and placed back in the incubator for further measurement. The concentrations of $OVA_{257-264}$ and LLO_{91-99} released from PLGA particles were measured by Pierce quantitative fluorometric peptide assay kit (Thermo Fisher Scientific, Waltham, MA).

2.6. Uptake of PLGA by BMDCs *in vitro*

On day 5 of BMDC culture, fluorescent PLGA (FITC-conjugated PLGA, Phosphorex) was added to the culture medium. After 2 hours or 2 days co-culture of fluorescent PLGA with BMDCs, BMDCs were fixed with 4% paraformaldehyde phosphate (Wako, Osaka, Japan) for 15 minutes at room temperature. After washing, early endosomes were stained with anti-mouse EEA-1 (early endosome antigen-1) mAb (Santa Cruz Biotechnology, Dallas, TX), and late endosomes were stained with anti-mouse LAMP-1 (lysosome associated membrane protein 1) mAb (Santa Cruz Biotechnology). As for the staining of acidic endosomes/lysosomes, BMDCs were stained with LysoTracker® Red (Thermo Fisher Scientific). Nuclei were stained with Hoechst. After staining, micrographs were taken with a fluorescent microscope (BZ-9000, KEYENCE, Osaka, Japan).

2.7. Lymphocytes proliferation assay *in vitro*

On day 5 of BMDC culture, CTL/Th-hybrid epitope long peptides and/or PLGA particles were added to the culture medium. Furthermore, splenocytes of OT-I and OT-II mice were collected, and cells were stained with 100 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) to evaluate cell proliferation. Then, BMDCs and splenocytes of OT-I and OT-II were co-cultured at 1:4:4 ratio for 2 days. On day 2 of co-culture, proliferations of CD8+ and CD4+ cells were measured by CFSE dilution assay with flow cytometry. Data analysis was performed using FlowJo v10.6.1 (BD, Flanklin Lakes, NJ).

2.8. Immunization of mice with peptides and PLGA particles, and *ex vivo* proliferation assay

On day 5 of BMDC culture, LLO-long peptides or PLGA particles with/without epitope peptides were added to the culture medium. 2×10^5 cells of antigen-loaded BMDCs were injected twice into flanks of BALB/c mice subcutaneously with a two-week interval. Three days after the second immunization, spleens were removed and splenocytes were incubated with 10 μM of CTL-epitope ($LLO_{91\text{-}99}$) and Th-epitope ($LLO_{215\text{-}226}$) peptides for 7 days. The concentrations of IFN-γ and Interleukin-4 (IL-4) in culture supernatant were evaluated by enzyme-linked immunosorbent assay (ELISA, R & D systems, Minneapolis, MN) and Cytometric Bead Array (BD Bioscience, San Jose, CA), respectively. Furthermore, splenocytes were labeled with 1 μM CFSE, and proliferations of CD8+ and CD4+ cells were measured by CFSE-dilution assay with flow cytometry.

2.9. *L. monocytogenes* challenge to vaccinated mice

For the evaluation of protection capacity of a novel vaccine, the immunized BALB/c mice were challenged intraperitoneally with 1×10^5 colony forming units (CFU) of *L.*

monocytogenes (10403S strain) (LD_{50} dose) four weeks after the last immunization. Bacterial numbers in the spleens were determined 72 hours after the challenge infection by plating 10- or 100-fold dilutions of tissue homogenates on brain heart infusion agar plates (Nissui Seiyaku, Tokyo, Japan).

2.10. Flow cytometry

Alexa Fluor 647-conjugated anti-CD8 mAb (53–6.7) was from BD Biosciences (Franklin Lakes, NJ) and PerCP-Cy5.5-conjugated anti-CD4 mAb (GK1.5) was from Biolegend (San Diego, CA). CFSE was from DOJINDO (Kumamoto, Japan). To remove dead cells, a Live/Dead fixable aqua dead cell stain kit was used at flow cytometry (Thermo Fisher Scientific).

2.11. Statistical analysis

Data from multiple experiments were expressed as the mean ± standard error of the mean (SEM). Data were analyzed using the Tukey-Kramer or Steel-Dwass test for the comparison of three or more groups, or using a Wilcoxon signed rank test for the comparison of two related datasets. Statistical analysis was performed using JMP 14.0. (SAS Institute, Cary, NC). All tests were two-sided and performed at the 0.05 significance level.

3. RESULTS

3.1. Characteristics of long-epitope/PLGA particles and release of peptides from PLGA particles

As shown in FIGURE 1, OVA-derived (FIGURE 1A) and LLO-derived (FIGURE 1B) CTL/Th- hybrid epitope long peptides were synthesized with glycine-linker. Several epitope

peptides-loaded PLGA particles were prepared with a double emulsion solvent evaporation method. Pictures of PLGA particles from scanning electron microscopy are shown in FIGURE 1C and 1D. Spheres of PLGA were uniform, but their sizes were variable. Their diameters were ranged from 0.45 to 6.44 μm with a median diameter of 1.38 μm (FIGURE 1E). As for peptide-releasing capacity, gradual releases of peptides from PLGA particles were confirmed (FIGURE 1F). At 2 days of incubation, 46.0% of OVA peptides ($OVA_{257-264}$) and 25.0% of LLO peptide (LLO_{91-99}) were released from PLGA particles *in vitro*. At 21 days of incubation, 76.5% of OVA peptide and 53.0% of LLO peptide were released. Compared to the release of OVA peptide, LLO peptide was more slowly released from PLGA.

3.2. PLGA particles exist in endosomes and lysosomes of BMDCs

On day 5 of BMDC culture, fluorescent PLGA particles (green) were added to the culture medium (FIGURE 2). Two hours after co-culture of fluorescent PLGA particles with BMDCs, PLGA particles were present in 29.6±1.56% of total BMDCs (FIGURE 2A). After 2 days, they were found in 48.8±2.26% of total BMDCs, and the significant increase in uptake was found between 2 hours and 2 days ($p < 0.01$). Next, localization of PLGA particles at 2 days co-culture was confirmed by fluorescent antibodies to early endosome (EEA-1 [red] which exclusively localizes to early endosomes), late endosome (LAMP-1 [red] which localizes late endosomes and lysosomes), lysosome (LysoTracker® [red] which selectively accumulates in cellular compartments with low internal pH), and Hoechst (blue) (FIGURE 2B). PLGA particles were found in vacuoles such as early endosome, late endosome, and lysosome, without escaping into cytosol.

3.3. Long peptide/PLGA-loaded BMDCs induce robust OVA-specific lymphocyte proliferation *in vitro*

To evaluate the antigen-specific lymphocyte-induction capacity of a novel long peptide/PLGA vaccine, OVA long peptide (FIGURE 1A) and transgenic mouse cells (OT-I and OT-II), which have OVA-specific TCR on CTL and Th, respectively, were used *in vitro*. Splenocytes of naïve OT-I and OT-II mice were collected, and cells were strained with CFSE. Then antigen-loaded BMDCs and OT-I and OT-II cells were mixed with a 1:4:4 ratio and co-cultured for 2 days. On day 2 of co-culture, proliferations of CD8+ (OT-I) and CD4+ (OT-II) cells were measured by CFSE-dilution assay. Representative results of CFSE-dilution assay are shown in FIGURE 3A, and percentages of divided cells in each group are shown in FIGURE 3B. The most robust proliferation of CD8+ cells was found in the long peptide/PLGA group (50.6±3.6%, FIGURE 3A and 3B). Furthermore, potent proliferation of CD4+ cells was also noted in both long peptide alone and long peptide/PLGA groups (41.4±9.2% and 48.1±2.4%, respectively). The most elevated IFN-γ level in culture supernatant was found in the long peptide/PLGA group (3.21±0.29 ng/mL, FIGURE 3C). These results of CD8+ and CD4+ cells, and IFN-γ in the long peptide/PLGA group reached statistical significance compared with those in other groups (FIGURE 3B and 3C). There were no significant differences in the production of IL-4 between groups (data not shown).

3.4. Long peptide/PLGA-loaded BMDC vaccination induces potent *L. monocytogenes*-specific lymphocyte-proliferation *ex vivo*

To evaluate the LLO-specific lymphocyte-induction capacity of a novel long peptide/PLGA vaccine, BMDCs loaded with LLO-long peptide/PLGA, LLO-long peptide alone, or LLO CTL- and Th-epitope short peptides/PLGA were administered *in vivo*. These antigen-loaded BMDCs were subcutaneously injected into the flanks of BALB/c mice on two occasions separated by a two-week interval (FIGURE 4A). Three days after the second immunization, spleens were removed and splenocytes were incubated with CTL-epitope ($LLO_{91\text{-}99}$) and

Th-epitope ($LLO_{215-226}$) peptides for 7 days. Representative results of CFSE-dilution assay are shown in FIGURE 4B. The most robust proliferation of CD8+ cells was found in the long peptide/PLGA group (43.5±6.2%, FIGURE 4B and 4C). The most robust proliferation of CD4+ cells was also noted in the long peptide/PLGA group (13.7±2.0%, FIGURE 4B and 4C). The most elevated IFN-γ level in culture supernatant was found in the long peptide/PLGA group (1.30±0.31 ng/mL, FIGURE 4D). These results of CD8+ and CD4+ cells, and IFN-γ in the long peptide/PLGA group reached statistical significance compared with those in other groups (FIGURE 4C and 4D). The production of IL-4 did not significantly differ between groups (data not shown). As for direct immunization without BMDCs, CD8+ and CD4+ cells were slightly proliferated in vaccination with the long peptide/PLGA (SUPPLEMENTARY FIGURE 1). Proliferation of CD4+ cells were significantly higher in vaccination with long peptide/PLGA compared to that with PLGA (p=0.020) or the mixture of the short epitope peptides/PLGA (p=0.032). However, these proliferations were obviously lower than those by BMDC-vaccination (FIGURE 4). In addition, the production of IFN-γ in culture supernatants was not detected in vaccination with the long peptide/PLGA without BMDCs.

3.5. Long peptide/PLGA-loaded BMDCs induce protective immunity against *L. monocytogenes* infection *in vivo*

Finally, we examined the vaccine's capability of inducing protective immunity against the intracellular pathogen. Antigen-loaded BMDCs were injected into the flanks of BALB/c mice subcutaneously on two occasions with a three-week interval (FIGURE 5A). For the evaluation of protection capacity of long peptide/PLGA vaccine against *L. monocytogenes*, the immunized mice were challenged intraperitoneally with LD_{50} dose of *L. monocytogenes* four weeks after the last immunization. The bacterial number in the spleens three days after the challenge infection was significantly lower in the LLO-long peptide/PLGA vaccination

group than in other groups ($149.5\times10^3\pm38.2$ CFU/spleen, $p < 0.05$, FIGURE 5B)

4. DISCUSSION

The present study is the first to demonstrate excellent efficacy of the novel vaccine taking advantages of CTL/Th-hybrid epitope long peptide/PLGA microparticles against intracellular pathogen infection. *In vitro*, OVA-hybrid epitope long peptide/PLGA-loaded DCs strongly induced OVA-specific CTL and Th proliferations as well as IFN-γ production. Furthermore, administration of DCs loaded with LLO-specific CTL/Th-hybrid epitope long peptide/PLGA microparticles elicited more robust LLO-specific CTL and Th proliferations and IFN-γ production than those of other vaccinations. Finally, this novel vaccine induced the most potent protective immunity against lethal *L. monocytogenes* infection.

Biodegradable PLGA micro- or nano-particles are safe carriers of antigenic peptides. PLGA particles protect peptides from degradation. Indeed, immunization with a 63 times-reduced dose of peptides encapsulated in PLGA particles was shown to induce a more robust CTL response than that of peptides in incomplete Freund's adjuvant [25]. Furthermore, immunization of PLGA particles containing antigenic proteins, which were synthesized by a double emulsion solvent evaporation method [26], can increase expression of costimulatory molecules on DCs, IL-12 production from DCs, and IL-2 secretion from CTL [24], indicating that PLGA particles have potent adjuvant activity. Moreover, PLGA particles also facilitate DC cross-presentation [17, 18]. In terms of the particle size, those with a diameter of 500 nm or more [27] or 1.1–1.4 μm for PLGA particles [28] efficiently induced DC cross-presentation. Our PLGA particles had a median diameter of 1.38 μm, which was appropriate for efficient induction of DC cross-presentation. The feature of controlled release of peptides from PLGA particles will contribute to sustained immune responses *in vivo*.

Antigen-specific CTL as well as Th1 play a pivotal role in establishing significant immunologic protection against intracellular bacteria, such as *Mycobacterium tuberculosis* [2, 3], and the close cooperation between CTL and Th1 is crucial [4, 7]. Several studies, including ours, showed some usefulness of CTL short peptide-loaded DC vaccines against intracellular pathogen infection [29, 30]. However, these DC vaccines only induced peptide-specific CTL, but not Th cells. When loading Th-epitope short peptides on DC vaccines, it is difficult to induce peptide-specific Th cells because of their poor accessibility to MHC class II molecules at the cell surface. In this regard, CTL/Th-hybrid epitope long peptides, containing both CTL- and Th-epitopes, were reported to successfully induce both antigen-specific CTL and Th1 [15, 22]. Unlike T-cell epitope short peptides, T-cell epitope long peptides are taken up mainly by APCs and can avoid immunologic tolerance induced by direct contact with T-cells and B-cells [13]. In addition, T-cell epitope long peptides facilitate DC cross-presentation [14] and lead to more potent antigen-specific CTL induction. In fact, vaccination with CTL/Th-hybrid epitope long peptide suppressed tumor growth of lung metastasis from colon cancer [15], and reduced tumor size of breast cancer [31]. Furthermore, CTL/Th-hybrid epitope long peptide induced more potent activation of antigen-specific CTL and Th1 [31] and longer lasting proliferative capacity of those cells [22] compared with a mixture of CTL- and Th-epitope short peptides. However, the efficacy of vaccines with CTL/Th-hybrid epitope long peptide against intracellular bacterial infection has not been explored. In this regard, the present study showed for the first time that DC vaccine with CTL/Th-hybrid epitope long peptides efficiently induced CTL and Th, which conferred potent protective immunity against intracellular bacterial infection, whereas those with a mixture of CTL- and Th-epitope short peptides did not.

Regarding processing of antigenic peptides, the kinetics may differ between CTL- and Th -epitope peptides [32, 33]. CTL-epitope peptide possibly remains in early endosome where a less

acidic environment exists [34], whereas processing of Th-epitope peptide and/or the peptide loading on MHC class II may occur in late endosome/lysosome or MIIC compartments [35]. In the present study, vaccination with CTL/Th-hybrid epitope long peptide/PLGA significantly induced more Th cells than the mixture of the short epitope peptides/PLGA (FIGURE 3, 4). Th-epitope short peptides may be vulnerable for endocytic proteases abundant in late endosome/lysosome, preventing efficient Th induction. In contrast, CTL/Th-hybrid epitope long peptides may be feasible for processing and binding to MHC class II molecules, as the peptide-binding groove of MHC class II molecules is open-ended. Therefore, Th-epitope peptide within the CTL/Th-hybrid epitope long peptides is not necessary to be processed precisely for MHC binding. Furthermore, Th-epitope peptide in the CTL/Th-hybrid epitope long peptides is able to bind nascent or recycled MHC class II molecules in early endosome, and then the peptides are trimmed by proteases ("bind first trim later" model) [36]. This binding to MHC class II protects Th-epitope determinant from proteases and may lead to significant induction of Th response [36]. These putative mechanisms are needed to be clarified in future studies.

In terms of DC cross-presentation, all of long peptide [14, 37], PLGA microparticle [17, 18, 28], and Th1-help [7] can enhance cross-presentation. Therefore, our novel vaccine, which consists of CTL/Th-hybrid epitope long peptide and PLGA particle and induces Th1, is likely to be ideal for inducing robust DC cross-presentation. In the present study, PLGA particles were found in endosome/lysosome but not in the cytosol. Phagosomal alkalinization is needed for effective cross-presentation in DC [34], and long peptides can be cross-presented via vacuolar pathway [5, 37]. Therefore, CTL/Th-hybrid epitope long peptide may be strong enough not to be completely degraded in phagosomes of DCs, and may be cross-presented via this pathway. In the present study, mice were immunized with BMDCs that were loaded with long peptide/PLGA. Moreover, direct immunization without BMDCs could not induce robust

immune response. Therefore, it seems that direct vaccination with long peptide/PLGA needs further potent adjuvant for future use.

In summary, we successfully developed the novel vaccine taking advantage of CTL/Th-hybrid epitope long peptide/PLGA microparticles, which could induce strong CTL/Th proliferation and IFN-γ production *in vitro* as well as *ex vivo*, and also confer potent protective immunity against lethal *L. monocytogenes* infection *in vivo*. These results provide important information about future development of efficient vaccines having the capability to induce both CTL and Th cells against intracellular pathogens.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

DATA AVAILABILITY

Research data are not shared.

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Figure legends

FIGURE 1 Structure of synthetic T-cell epitope long peptides, morphology and size of PLGA particles, and peptide-releasing profile from PLGA particles. OVA-derived CTL/Th-hybrid epitope long peptide composed of CTL ($OVA_{257-264}$) and Th ($OVA_{323-339}$) epitopes with glycine-linker (A). LLO-derived CTL/Th-hybrid epitope long peptide composed of CTL (LLO_{91-99}) and Th ($LLO_{215-226}$) epitopes with glycine-linker (B). Pictures of PLGA particles by scanning electron microscopy (SEM) showing spheres of different diameters (C: low- and D: high-power field). Each scale bar represents 10 μm (C) or 0.5 μm (D), respectively. Spheres of PLGA were uniform, but their sizes were variable. Distribution of PLGA particle sizes is described (E). Their diameters ranged from 0.45 to 6.44 μm with a median diameter of 1.38 μm. To evaluate the release profile of peptides ($OVA_{257-264}$ and LLO_{91-99}) from PLGA particles *in vitro*, 0.2 mg of the particles was resuspended in PBS under agitation at 37°C. At each time point, the concentrations of $OVA_{257-264}$ (solid line) and LLO_{91-99} (dotted line) in supernatant were measured. Compared to the release of OVA peptide, LLO peptide was more slowly released from PLGA. At 2 days of incubation, 46.0% of OVA peptides ($OVA_{257-264}$) and 25.0% of LLO peptide (LLO_{91-99}) were released from PLGA particles *in vitro*. At 21 days of incubation, 76.5% of OVA peptide and 53.0% of LLO peptide were released (F). Data are presented as percentages of the total amount of the peptide initially encapsulated in the particles. Error bars represent SEM in three independent experiments.

FIGURE 2 Uptake and localization of PLGA particles in BMDCs. On day 5 of BMDC culture, fluorescent PLGA particles (green) were added to culture medium. After 2 hours co-culture of fluorescent PLGA particles with BMDCs, PLGA particles were found in 29.6 ±

1.6% of total BMDCs (A). After 2 days, they were found in 48.8±2.3% of total BMDCs and a significant increase in the uptake was found between 2 hours and 2 days ($p < 0.01$). Localization of PLGA particles in BMDCs at 2 days co-culture were confirmed by fluorescent antibodies to early endosome (EEA-1 [red] which exclusively localizes in early endosomes), late endosome (LAMP-1 [red] which localizes in late endosomes and lysosomes), lysosome (LysoTracker® [red] which selectively accumulates in cellular compartments with low internal pH), and Hoechst (blue). Representative fluorescent and bright-field microscopic images of BMDCs are shown (B, ×10000). PLGA particles are found in early endosome, late endosome, and lysosome, but not in the cytosol. Insets show magnified images and red arrows indicate PLGA particles. Error bars represent SEM in three independent experiments. ** $p <$ 0.01.

FIGURE 3 Long peptide/PLGA-loaded BMDCs induced robust OVA-specific lymphocyte proliferation *in vitro*. Splenocytes of naïve OT-I and OT-II mice, which have OVA-specific T-cell receptor on CTL and Th, were collected. Then peptide-loaded BMDCs and OT-I and OT-II cells were co-cultured for 2 days. On day 2 of co-culture, proliferations of CD8+ (OT-I) and CD4+ (OT-II) cells were measured by CFSE-dilution assay. Representative results of CFSE-dilution assay (A) and percentages of divided cells in each group are shown (B). The most robust proliferation of CD8+ cells was found in long peptide/PLGA group (50.6±3.6%, A and B). Furthermore, potent proliferation of CD4+ cells was found in both long peptide alone and the long peptide/PLGA group (41.4±9.2% and 48.1±2.4%, respectively, A and B). The most elevated IFN-γ level in culture supernatants was found in long peptide/PLGA group (3.21±0.29 ng/mL, C). Error bars represent SEM in three independent experiments. ** $p <$ 0.01. Long-p: CTL/Th-hybrid epitope long peptide, CTL-p: CTL-epitope short peptide, Th-p: Th-epitope short peptide.

FIGURE 4 *L. monocytogenes* listeriolysin O (LLO)-derived Long peptide/PLGA-loaded BMDCs induced potent lymphocyte-proliferation *ex vivo*. LLO-peptides-loaded BMDCs were injected into flank of BALB/c mice subcutaneously twice with a two-week interval *in vivo* (A). Three days after second immunization, spleens were removed and the splenocytes were incubated with CTL-epitope (LLO_{91-99}) and Th-epitope ($LLO_{215-226}$) peptides for 7 days. Representative results of CFSE-dilution assay (B) and percentages of divided cells (C) in each group are shown. The most robust proliferation of CD8+ cells was found in the long peptide/PLGA group (43.5±6.2%, B and C). In addition, the most robust proliferation of CD4+ cells was also found in the long peptide/PLGA group (13.7±2.0%, B and C). The most elevated IFN-γ level in culture supernatant was found in the long peptide/PLGA group (D). Error bars represent SEM in three independent experiments. *$p < 0.05$, **$p < 0.01$. Long-p: CTL/Th-hybrid epitope long peptide, CTL-p: CTL-epitope short peptide, Th-p: Th-epitope short peptide.

FIGURE 5 Long peptide/PLGA-loaded BMDCs induced protective immunity against *L. monocytogenes* infection *in vivo*. *L. monocytogenes* listeriolysin O (LLO)-derived antigen-loaded BMDCs were injected into flanks of BALB/c mice subcutaneously twice with a three-week interval (A). For the evaluation of protection capacity of long peptide/PLGA vaccine against *L. monocytogenes*, the immunized mice were challenged intraperitoneally with *L. monocytogenes* four weeks after the last immunization. Bacterial numbers in the spleens three days after the challenge infection were significantly lower in the long peptide/PLGA group than in other groups (B, $p < 0.05$, 149,500±38,200 CFU). Error bars represent SEM in seven independent experiments. *$p < 0.05$. Long-p: CTL/Th-hybrid epitope long peptide, CTL-p: CTL-epitope short peptide, Th-p: Th-epitope short peptide, CFU:

colony forming units.

SUPPLEMENTARY FIGURE 1 Direct immunization with *L. monocytogenes* listeriolysin O (LLO)-derived Long peptide/PLGA did not induce potent lymphocyte-proliferation *ex vivo*. LLO-peptides/PLGA was injected into flank of BALB/c mice subcutaneously twice with a one-week interval *in vivo* (A). Seven days after second immunization, spleens were removed and the splenocytes were incubated with CTL-epitope (LLO_{91-99}) and Th-epitope ($LLO_{215-226}$) peptides for 7 days. Representative results of CFSE-dilution assay (B) and percentages of divided cells (C) in each group are shown. The most robust proliferation of CD8+ cells was found in the long peptide/PLGA group (6.2±4.2%, B and C). In addition, the most robust proliferation of CD4+ cells was also found in the long peptide/PLGA group (3.0±1.1%, B and C). Proliferation of CD4+ cells were significantly higher in vaccination with long peptide/PLGA compared to that with PLGA alone ($p=0.020$) or the mixture of the short epitope peptides/PLGA ($p=0.032$). However, these proliferation rates were clearly lower than those by BMDC-vaccination (FIGURE 4). IFN-γ in culture supernatants was not detected across the groups. Error bars represent SEM in three independent experiments. $*p < 0.05$. Long-p: CTL/Th-hybrid epitope long peptide, CTL-p: CTL-epitope short peptide, Th-p: Th-epitope short peptide.

FIGURE 1

A **OVA long peptide:**

SIINFEKLGGGGGISQAVHAAHAEINEAGR

(OVA 257-264:CTL-epitope) **(OVA 323-339:Th-epitope)**

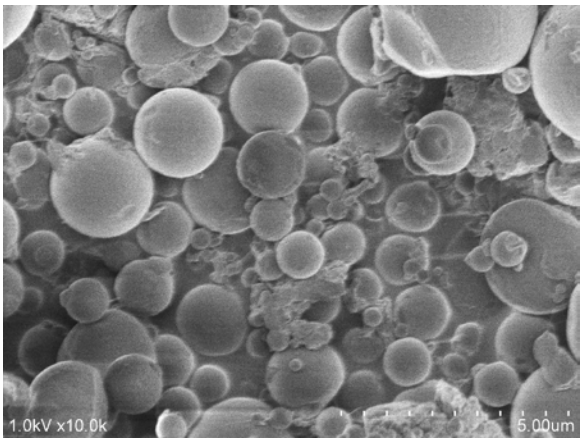
B **LLO long peptide:**

GYKDGNEYIGGGGGSQLIAKFGTAFK

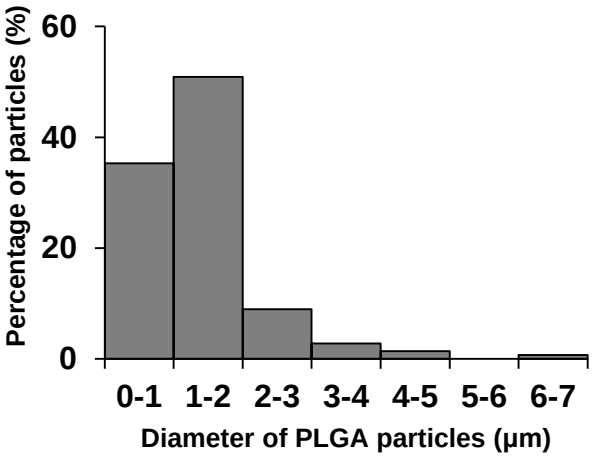
(LLO 91-99: CTL-epitope) **(LLO 215-226: Th-epitope)**

C

D



E



F

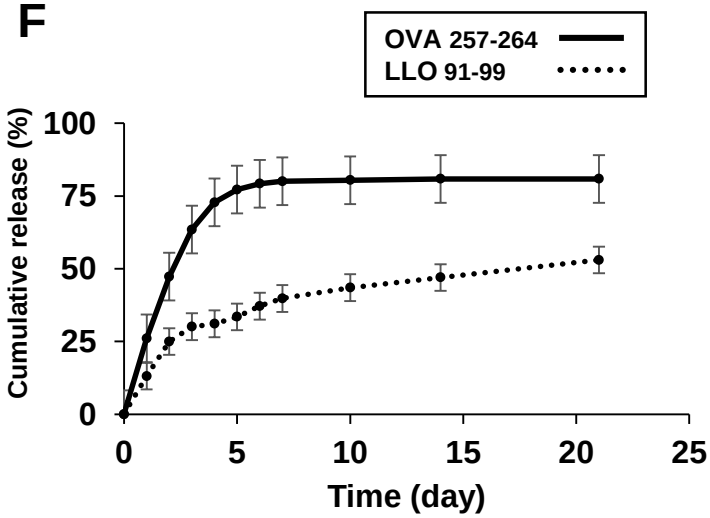
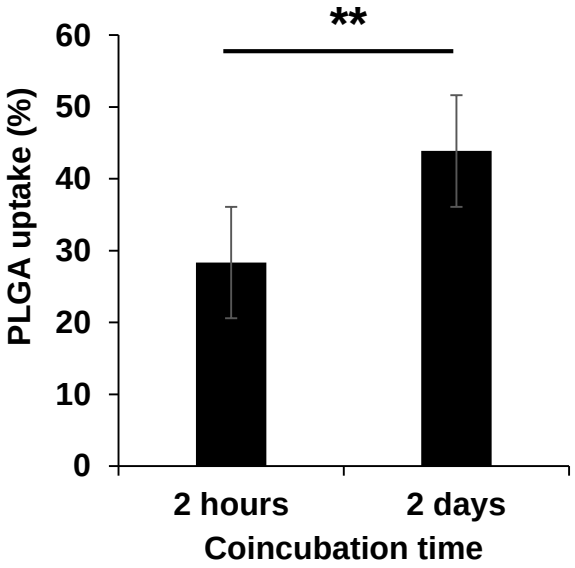


FIGURE 2

A


**
60
50
40
30
20
10
0
PLGA uptake (%)
2 hours
2 days
Coincubation time

B

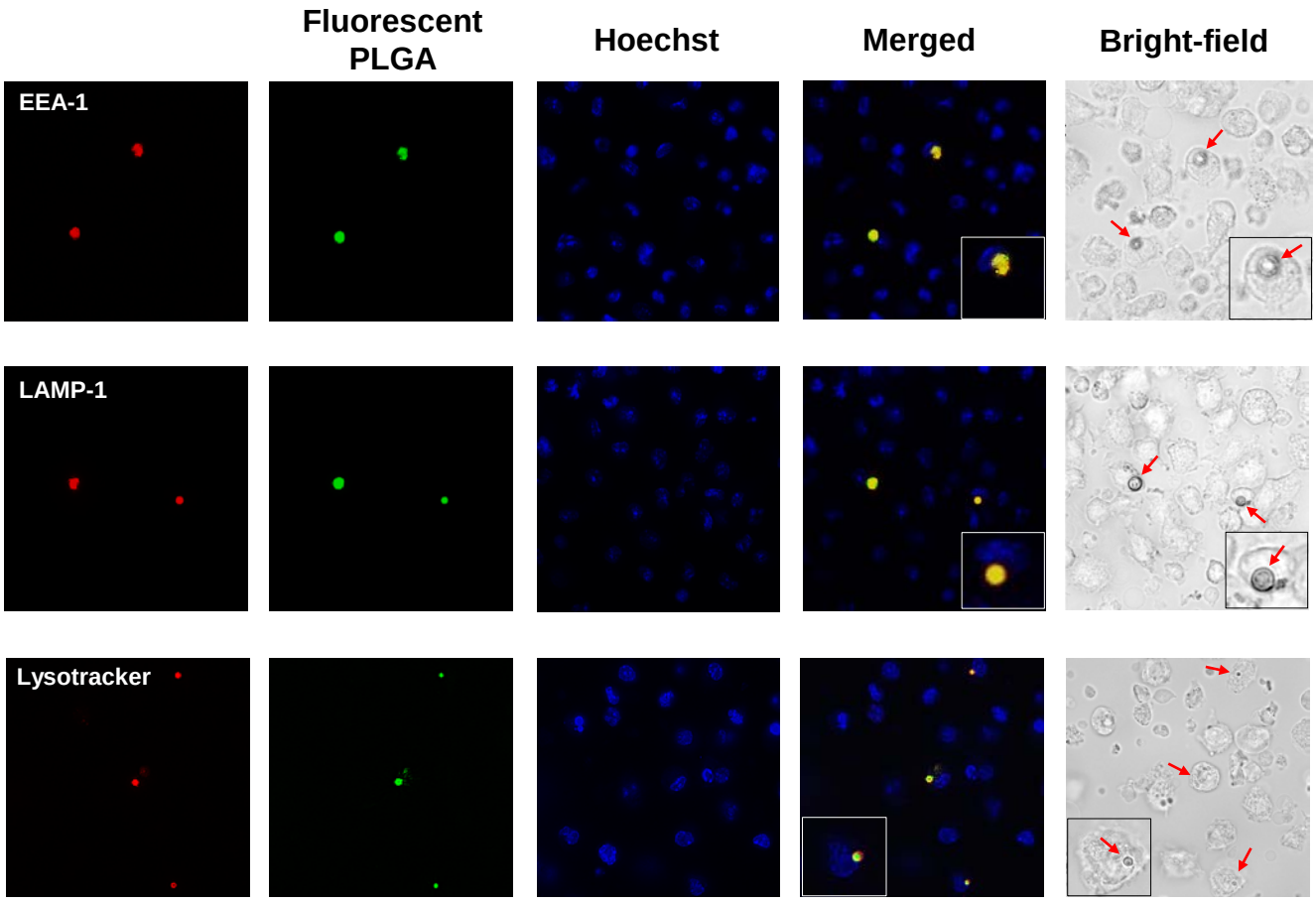

Fluorescent PLGA
Hoechst
Merged
Bright-field
EEA-1
LAMP-1
Lysotracker

FIGURE 3

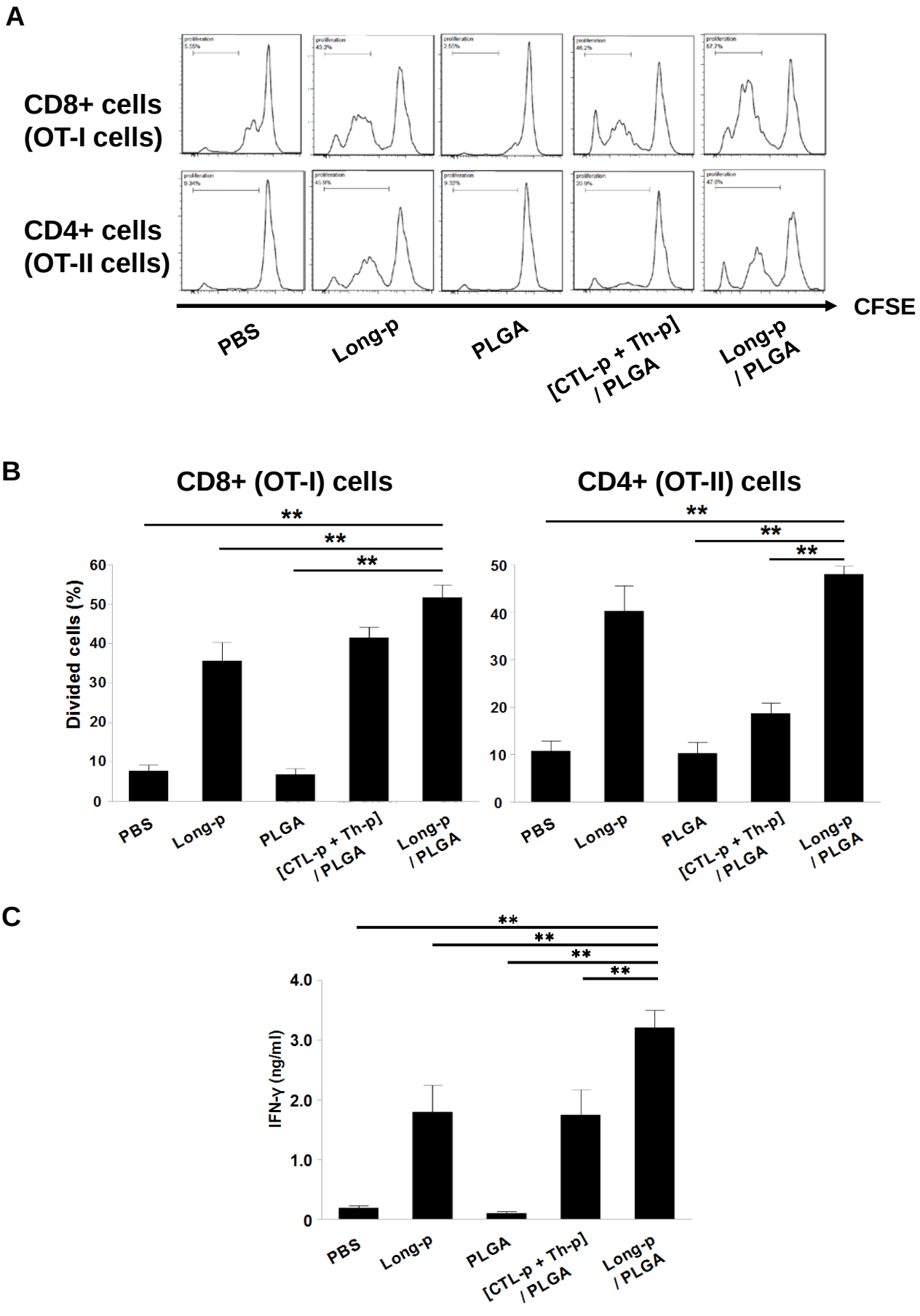

A
CD8+ cells
(OT-I cells)
CD4+ cells
(OT-II cells)
CFSE
PBS
Long-p
PLGA
[CTL-p + Th-p]
/ PLGA
Long-p
/ PLGA
B
CD8+ (OT-I) cells
CD4+ (OT-II) cells
Divided cells (%)
**
PBS
Long-p
PLGA
[CTL-p + Th-p]
/ PLGA
Long-p
/ PLGA
C
IFN-γ (ng/ml)
4.0
3.0
2.0
1.0
0

FIGURE 4

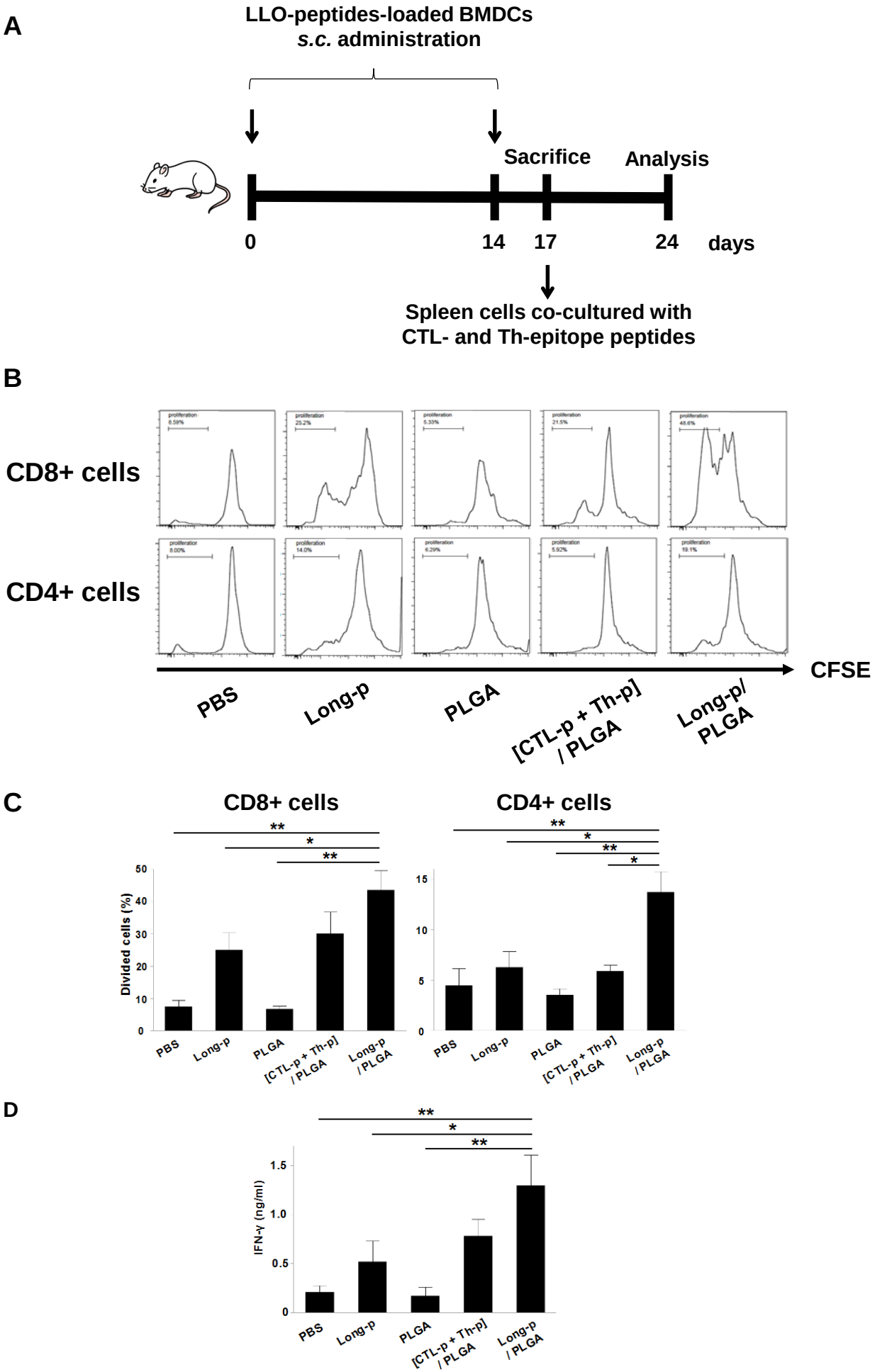
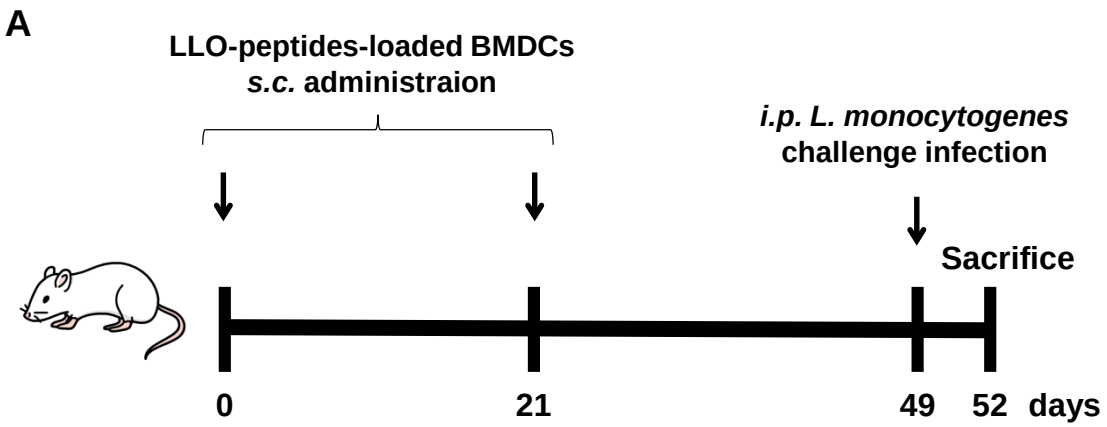
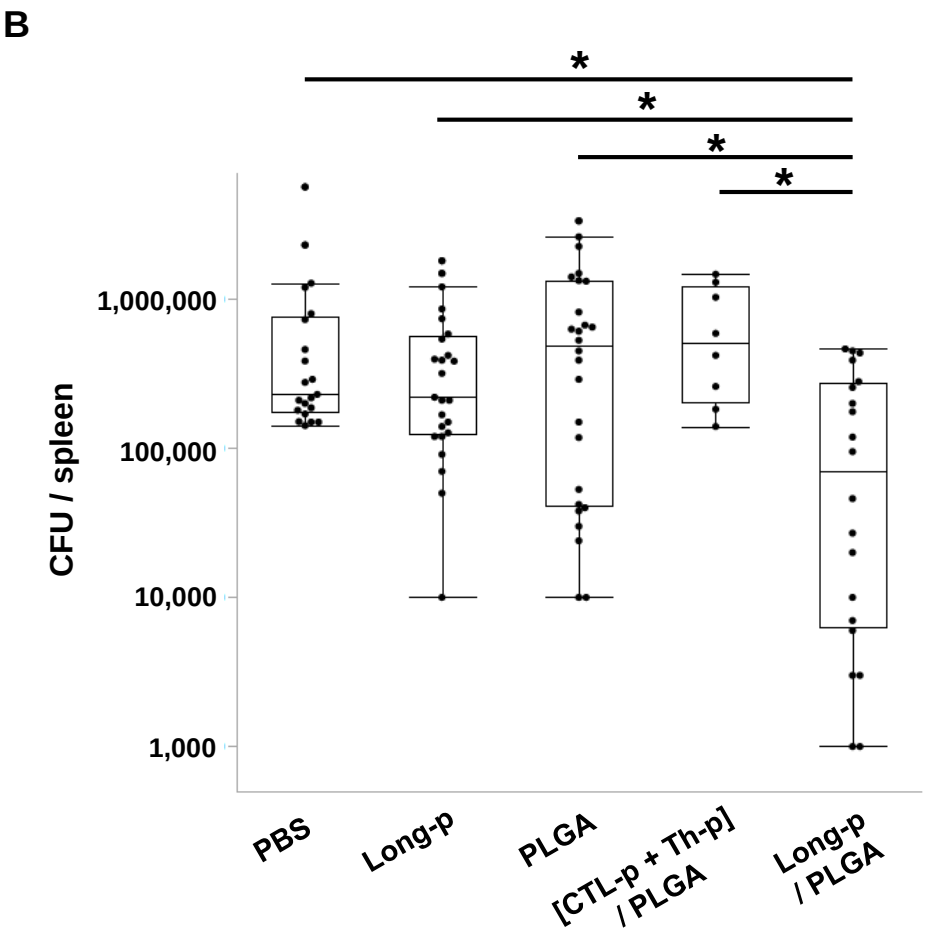

A
LLO-peptides-loaded BMDCs
s.c. administration
Sacrifice
Analysis
0
14
17
24
days
Spleen cells co-cultured with
CTL- and Th-epitope peptides
B
CD8+ cells
CD4+ cells
CFSE
PBS
Long-p
PLGA
[CTL-p + Th-p]
/ PLGA
Long-p/
PLGA
C
CD8+ cells
CD4+ cells
Divided cells (%)
PBS
Long-p
PLGA
[CTL-p + Th-p]
/ PLGA
Long-p
/ PLGA
D
IFN-γ (ng/ml)

FIGURE 5


A
LLO-peptides-loaded BMDCs
s.c. administraion
i.p. L. monocytogenes
challenge infection
Sacrifice
0
21
49
52
days


B
*
*
*
*
CFU / spleen
1,000,000
100,000
10,000
1,000
PBS
Long-p
PLGA
[CTL-p + Th-p]
/ PLGA
Long-p
/ PLGA

SUPPLEMENTARY FIGURE 1