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Dendritic Cell Membrane Vesicles for Activation and Maintenance of Antigen-specific T Cells

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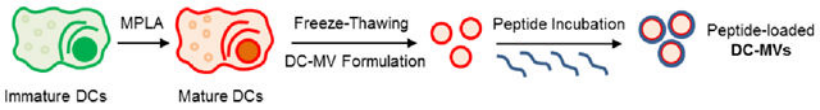
Abstract

Cell membranes have recently gained attention as a promising drug delivery system. Here, dendritic cell membrane vesicles (DC-MVs) are examined as a platform to promote T cell responses. Nano-sized DC-MVs are derived from dendritic cells (DCs) pre-treated with monophosphoryl lipid A (MPLA), an FDA-approved immunostimulatory adjuvant. These "mature" DC-MVs activate DCs *in vitro* and increase their expression of co-stimulatory markers. DC-MVs also promote cross-priming of antigen-specific T cells *in vitro*, increasing their survival and CD25 expression. In addition, these mature DC-MVs potently augment the expansion of adoptively transferred CD8+ T cells *in vivo*, generating 2-4 fold higher frequency of antigen-specific T cells, compared with other control formulations, including "immature" DC-MVs obtained without the MPLA pre-treatment. Taken together, these results suggest that DC-MVs are an effective delivery platform for T cell activation and may serve as a potential delivery system for improving adoptive T cell therapy.

Graphical Abstract



Nano-sized dendritic cell membrane vesicles (DC-MVs) are examined as a platform to promote T cell responses. DC-MVs activate DCs and promote cross-priming of CD8+ T cells *in vitro*. DC-MVs potently amplify the expansion of adoptively transferred CD8+ T cells *in vivo*. DC-MVs may serve as a potential peptide delivery system for augmenting adoptive T cell therapy.

Keywords

membrane vesicle; vaccine; peptide antigen; T cell activation

Cancer immunotherapy is transforming the current landscape of oncology.[1] Adoptive T cell therapy has recently shown great promise and produced effective clinical responses, leading to clinical approval for blood cancers.[2] Immune checkpoint blockade designed to release the brakes from the immunosuppressive tumor environment allows for anti-tumor therapeutic effects.[3] At the same time, advances in neo-antigen characterization in tumors has pushed for readily-synthesized and potent peptide-based vaccination.[4,5] These discoveries and a tremendous amount of work over the past decade had led to the development of effective cancer vaccines evaluated in clinical trials.[6] However, induction of effective immunity is often dependent on appropriate antigen delivery methods for optimal responses, with peptide-based vaccines being particulary susceptible to forming depots at the site of administration leading to diminished potency.[7]

In this study, we examined cell-derived vesicles for antigen delivery to promote antigen-specific T cell responses. Utilizing cell membrane preparation technologies that we and others have reported,[8–10] we generated dendritic cell membrane vesicles (DC-MVs) from pre-activated antigen-presenting cells (APCs) (Figure 1A). Here, we show that DC-MVs can be effectively loaded with antigen peptides and promote activation of antigen-specific T cells *in vitro*. We also demonstrate their potency to expand adoptively transferred T cells *in vivo*. Our results suggest that DC-MVs is a promising platform for augmenting adoptive T cell therapy and improving peptide-based cancer vaccination.

To generate DC-MVs, we first obtained DCs from bone marrow of C57BL/6 mice with the use of granulocyte-macrophage colony-stimulating factor.[11] Immature bone marrow-derived dendritic cells (BMDCs) were lysed by freeze-thaw cycles and mild probe-tip sonication. After removing large debris and organelles via centrifugation, the resulting lysate samples were incubated with 20 mM $CaCl_2$ for 1 hour to promote fusion and aggregation of cellular membranes, which allowed for isolation of DC-MVs with table-top centrifugation. We sought to pre-activate DCs before generating DC-MVs and studied their impact on the subsequent T cell cross-priming. Specifically, we pre-activated BMDCs with monophosphoryl lipid A (MPLA), an FDA-approved immunostimulatory Toll-like receptor 4 agonist.[12,13] We first confirmed upregulation of co-stimulatory markers, including CD80 and CD86, in whole cell lysate of MPLA-treated DCs by Western blotting (Figure 1B). Immature DCs without any MPLA treatment (NO TX) exhibited minimal expression of CD80 or CD86. In contrast, pre-treatment of DCs with MPLA increased the expression levels of co-stimulatory markers, in particular, CD86, on DC-MVs regardless whether BMDCs were harvested from culture dishes either by Accutase treatment and a cell scraper (MPLA) or by pipetting (MPLA-S) (Figure 1B). Based on these results and high yield of total protein (80%) from the Accutase-based harvest, we proceeded with this method for the source for MPLA-activated DC-MVs, which is henceforth termed (MPLA)DC-MVs. Dynamic light scattering analysis indicated that (MPLA)DC-MVs had an average hydrodynamic size of 130 ± 4 nm and a polydispersity index of 0.17 ± 0.01.

Next, we examined the ability of DC-MVs to present antigen peptides directly to T cells and promote their activation and proliferation through engagement with T cell receptor (TCR, signal 1) and CD28 (signal 2). We employed a model antigen peptide SIINFEKL, an immunodominant MHC-I epitope from ovalbumin (OVA). To examine the interactions

between DC-MVs and antigen-specific T cells, we performed the commonly used carboxyfluorescein succinimidyl ester (CFSE) dilution assay[14] with SIINFEKL-specific CD8α+ T cells obtained from OT-I transgenic mice with the exception that CD8α+ T cells were directly induced with DC-MVs without the presence of any intact APCs. Using the standard 50,000 cells/well seeding density of CD8α+ OT-I T cells, we observed that SIINFEKL peptide alone promoted CFSE dilution of T cells; however, the number of OT-I T cells surviving at the end of the three day culture was low, with minimal expression of CD25, which is a subunit of the IL-2 receptor and a late marker of TCR-dependent T cell activation (Figure 1C). This may be due to direct peptide binding and epitope presentation in the context of MHC-I on the T cell surface itself, resulting in swift T cell cross-priming and cross-killing, a phenomenon termed fratricide.[15] In contrast, we observed strong proliferation of T cells with the increased expression of CD25 when we cultured CD8α+ OT-I T cells in the presence of 10 ng/ml SIINFEKL and immature DC-MVs (Figure 1C). For these *in vitro* studies, we added DC-MV formulations directly to SIINFEKL-containing media without column purification in order to ensure equivalent antigen dose without any variation across all groups. Notably, DC-MVs pre-activated with MPLA further amplified T cell responses; (MPLA)DC-MV-SIINFEKL significantly enhanced T cell survival, compared with the DC-MV-SIINFEKL group and the SIINFEKL control group ($p < 0.0001$, at 10 ng/mL SIINFEKL concentration, Figure 1C). In addition, (MPLA)DC-MV-SIINFEKL significantly improved the CD25 expression on CD8α+ OT-I T cells, compared with either control groups ($p < 0.0001$, at 10 ng/mL SIINFEKL concentration, Figure 1C). We also confirmed these results using a lower seeding density of OT-I T cells (10,000 cells/well) aimed to limit direct cell-cell interactions (Figure 1D). Taken together, these results suggested that MPLA pre-treatment increased the expression of co-stimulatory markers on DC-MVs and improved their efficacy to cross-prime antigen-specific T cell responses *in vitro*.

While direct stimulation of T cells may be possible in the controlled *in vitro* conditions, it may be difficult to achieve this within the complex *in vivo* environment. To address this, we examined if DC-MVs taken up by APCs can indirectly enhance T cell activation. Also, we sought to compare DC-MVs pre-activated with MPLA or CpG (a TLR-9 agonist based on DNA oligonucleotide with unmethylated CpG motifs[16]). For this particular experiment, we prepared DC-MV formulations pre-incubated with SIINFEKL overnight, followed by column chromatography. This ensured that unbound antigen was separated out, thus eliminating the possibility of free SIINFEKL directly binding BMDCs and activating T cells. BMDCs were pulsed with various SIINFEKL-loaded DC-MV formulations overnight, washed extensively, and incubated with CFSE-labeled OT-I T cells for three days, followed by flow cytometry-based analysis. BMDCs incubated with (MPLA)DC-MV-SIINFEKL increased proliferation (Figure 2A-C) and CD25 expression of OT-I T cells in a dose-dependent manner (Figure 2D). (MPLA)DC-MV-SIINFEKL significantly enhanced T cell proliferation, compared with unactivated DC-MVs at 10 μg/ml protein dose of DC-MVs ($p < 0.01$), and there was a trend (although no statistical difference) for increased T cell proliferation with (MPLA)DC-MV-SIINFEKL, compared with (CpG)DC-MV-SIINFEKL (Figure 2B).

We also examined whether introducing poly(ethylene glycol) (PEG) on the surfaces of DC-MVs affected their efficacy to activate T cells. To produce PEGylated DC-MVs, we incubated DC-MVs with 10 mg/ml DSPE-PEG in 100 mM EDTA solution and used a mild water bath sonication,[10] which promoted surface coating with PEG while removing excess calcium added during the purification of DC-MVs as detailed above.

PEGylated (MPLA)DC-MVs significantly improved T cell proliferation, compared with PEGylated DC-MVs from either unactivated or CpG-treated DCs at the 10 μg/ml dose ($p < 0.0001$ and $p < 0.01$, respectively, Figure 2B), and this trend was observed at DC-MV concentrations ranging from 2 to 10 μg/ml (Figure 2C-D). However, at higher concentration of 50 μg/ml, the PEGylated DC-MV formulations exhibited loss of bioactivity (Figure 2C-D), supposedly by interfering with antigen uptake and presentation. Comparing PEGylated versus non-PEGylated DC-MVs, we did not observe any statistical differences within each adjuvant-induced stimulation condition, except for CpG-treated DC-MVs that exhibited decreased T cell proliferation after PEGylation ($p < 0.05$, Figure 2A-B), potentially due to PEG-mediated interference of vesicle-cell interactions. Overall, these results demonstrated that DC-MVs could activate T cells via an indirect pathway of APC-mediated uptake and antigen presentation to T cells. Based on these results, we chose to focus on (MPLA)DC-MVs without PEGylation for the subsequent studies.

As naturally-produced membrane vesicles are known to transduce cell-to-cell signals,[17,18] we sought to determine if the artificially produced DC-MVs can activate live DCs *in vitro*. BMDCs were pulsed with various DC-MV formulations and analyzed for activation markers, including CD40 and CD80, via flow cytometry. (MPLA)DC-MVs significantly upregulated both CD40 and CD80 on BMDCs, compared with unactivated DC-MVs ($p < 0.0001$ and $p < 0.001$, respectively, at 200 μg/ml dose, Figure 3A), demonstrating their potency to activate DCs.

Next, we examined the ability of DC-MVs to load SIINFEKL peptide in an MHC-I haplotype-specific manner. As SIINFEKL has a high affinity for H-2K^b from C57BL/6 mouse strain,[19] we examined the amount of SIINFEKL peptide bound on DC-MVs derived from C57BL/6 (H-2K^b, H-2D^b) and compared to that from BALB/c (H-2K^d, H-2D^d, H-2L^d) as the control group. To quantify peptide loading, we utilized SIINFEKL peptide with a covalently bound fluorophore (FITC) to the lysine amino acid of the peptide (SIINFEK(FITC)L), which can effectively bind to H-2K^b MHC-I.[19,20] We generated (MPLA)DC-MVs or unstimulated DC-MVs and incubated them with SIINFEK(FITC)L at various membrane protein concentrations, followed by desalting column chromatography to remove any unbound peptide. We observed a similar amount of SIINFEKL peptide loaded onto DC-MVs regardless of the MHC-I haplotypes (C57BL/6 versus BALB/c) or pre-activation status with MPLA (Figure 3B). This outcome suggests that the peptide is likely loaded onto DC-MVs via non-specific interactions; however, we cannot rule out the possibility that a small fraction of peptide is specifically bound to and presented by H-2K^b MHC-I molecules on DC-MVs due to small quantity of pMHC-I complexes in DC-MV formulations.

Based on our *in vitro* results, we next evaluated if DC-MVs can promote T cell activation *in vivo*. We utilized adoptive cell transfer of OT-I T cells to provide a basal, equivalent frequency of antigen-specific T cells within each animal. On day 0, OT-I CD8α+Thy1.1+ T cells (5×10^5 cells per animal) were adoptively transferred via intravenous administration into recipient, congenic Thy1.2+ C57BL/6 mice, and on day 1, the animals were immunized with various DC-MV formulations. On day 6, peripheral blood samples were analyzed for the frequency of Thy1.1+ T cells. DC-MV formulations were incubated with 10 μg SIINFEKL and used directly without column purification in order to ensure equivalent antigen dose without any variation across all groups. Subcutaneous administration of soluble SIINFEKL with or without MPLA resulted in a weak expansion of adoptively transferred OT-I T cells, with 15–20% Thy1.1+ T cells among CD8α+ T cells (Figure 3C). Unactivated DC-MV-SIINFEKL did not enhance the expansion of OT-I T cells, compared with the soluble controls. In contrast, (MPLA)DC-MV-SIINFEKL derived from C57BL/6 mice markedly improved the expansion of OT-I T cells, achieving 4.1-fold, 2.4-fold, and 2.1-fold increases, compared with the SIINFEKL, SIINFEKL+MPLA, and DC-MV-SIINFEKL groups ($p < 0.05$, $p < 0.01$, and $p < 0.05$, respectively, Figure 3C), thus demonstrating that MPLA-induced pre-activation of BMDCs played a critical role in T cell activation by (MPLA)DC-MVs. Notably, (MPLA)DC-MV-SIINFEKL derived from BALB/c mice also induced robust expansion of OT-I T cells, compared with other control groups (Figure 3C); however, there was no statistical difference between (MPLA)DC-MV-SIINFEKL formulations derived from C57BL/6 or BALB/c mice.

Taken together, these results suggest that DC-MVs are an effective delivery platform for peptide-based vaccines and may serve as potential nanomaterials for cancer immunotherapy. In particular, these nano-sized DC vesicles are expected to effectively drain to target lymphatic tissues and may promote the maintenance of adoptive T cell therapies through the functional presentation of co-stimulatory markers or antigen presentation by APCs. Additionally, DC-MVs generated from a patient's blood-sourced cells are expected to be fully biocompatible without triggering anti-vector immunity, thus providing a new material platform for cancer immunotherapy.

Experimental Section

DC-MV Preparation and Peptide Loading:

BMDCs were generated as previously described.[11] DC membrane vesicles (DC-MVs) were generated from sonicated cell lysate, following the removal of large debris and organelles via centrifugation (10,000 × g, 10 minutes). Lysate samples were adjusted to 6 mg/ml concentration and incubated with 20 mM $CaCl_2$ for 1 hour to promote fusion and aggregation of membranes, which allowed for washing with table-top centrifugation (20,000 × g, 5 minutes). Where indicated, DC-MVs were then resuspended with mild water bath sonication in 10 mg/ml DSPE-PEG in 100 mM EDTA solution in PBS, which promoted surface coating with PEG and removal of excess calcium by chelation. Finally, DC-MVs were passed through PBS-equilibrated Zeba desalting column to remove excess EDTA, calcium, and DSPE-PEG to generate PEGylated DC-MVs. SIINFEKL or fluorescently labeled SIINFEK(FITC)L peptides (Genscript) were loaded at 100 μg/ml onto DC-MVs by

incubation at 37°C at varying concentrations of DC-MVs (10.0, 2.5, and 1.0 mg/ml in PBS). DC-MV concentrations were measured by Pierce BCA assay (ThermoFisher). Peptide loading efficiency was determined after samples were passed two times through 40 kDa Zeba desalting column and quantified by a microplate-based fluorescence assay.

T Cell Proliferation In Vitro:

T cell proliferation was assessed using SIINFEKL-specific primary T cells obtained from OT-I transgenic mice. Briefly, spleens from 6- to 12-week old OT-I transgenic mice were harvested and processed into single cell suspension. Red blood cells were removed by three-minute incubation with ACK lysis buffer (Gibco) and CD8α+ T cells were separated using a CD8α+ T cell negative selection kit (StemCell Technologies). Cells were then labeled with 1 μM CFSE solution by incubating at 37°C for ten minutes and, after washing, resuspended in complete T cell media (RPMI supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol, 1 mM HEPES buffer, and 1X non-essential amino acid solution (Gibco)). Cells were then plated in 96-wells at 50,000 or 10,000 cells/well and treated with 0.01 to 10 ng/ml SIINFEKL peptide with or without 50 μg/ml of DC-MVs. After three days of culture, T cells were collected, washed, blocked with FACS buffer containing anti-CD16/32 antibodies, and stained with anti-CD8α, anti-CD25, and DAPI, followed by flow cytometry. The results were analyzed by FlowJo software.

Animal Studies:

Animals were cared for following the federal, state, and local guidelines. All work performed on animals was in accordance with and approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan, Ann Arbor. OVA-specific, Thy1.1+ OT-I CD8α+ T cells were obtained as described above, and 5×10^5 cells were adoptively transferred into naïve congenic Thy1.2+ C57BL/6 mice (female, 6–8 weeks, Envigo, USA) via intravenous tail vein injection. One day after the transfer, the animals were immunized with SIINFEKL peptide (10 μg per mouse) with or without DC-MVs (250 μg protein per mouse). After 5 days, peripheral blood samples were obtained using submandibular bleeds and red blood cells removed via ACK lysis to yield peripheral blood mononuclear cells (PBMCs). Samples were processed for flow cytometry by washing, blocking CD16/32 Fc receptor, and staining with anti-CD8α and anti-Thy1.1. Cells were then resuspended in FACS DAPI solution and examined via flow cytometry.

Statistical analyses:

Sample sizes were chosen based on preliminary data from pilot experiments and previously published results in the literature. Statistical analysis was performed with Prism 6.0 software (GraphPad Software) by two-way ANOVA with Tukey's comparisons post-test, as indicated. Data were normally distributed and variance between groups was similar. All values are reported as means ± SD. Statistical significance is indicated as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

Acknowledgements and financial conflict of interest

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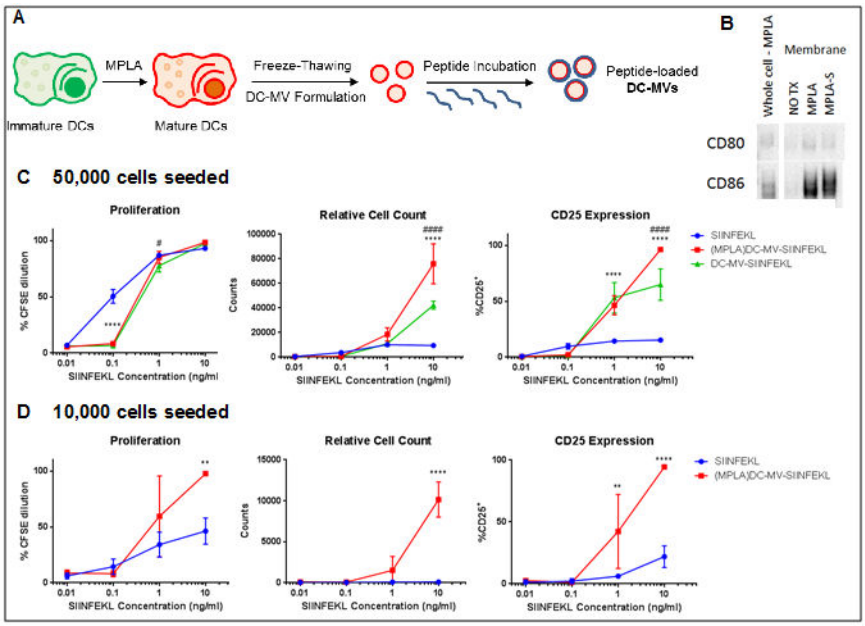

Figure 1. DC-MVs directly promote T cell activation and proliferation *in vitro*.

A. Schematic demonstrating preparation of DC-MVs. **B**. Western Blot analysis of MPLA-activated DCs and activation marker-enriched membrane fraction. **C-D**. Proliferation and activation of OT-I T cells are shown after seeding T cells at 50,000 or 10,000 cells per well in the presence of SIINFEKL alone or SIINFEKL mixed in with DC-MVs. Mean ± SD are shown. Statistical analysis was performed using two-way ANOVA comparison with Tukey's multiple comparison test comparing (MPLA)DC-MV-SIINFEKL to either DC-MV-SIINFKEL ($^{\#}p < 0.05$, $^{\#\#\#\#}p < 0.0001$) or SIINFEKL alone ($^{**}p < 0.01$, $^{****}p < 0.0001$).

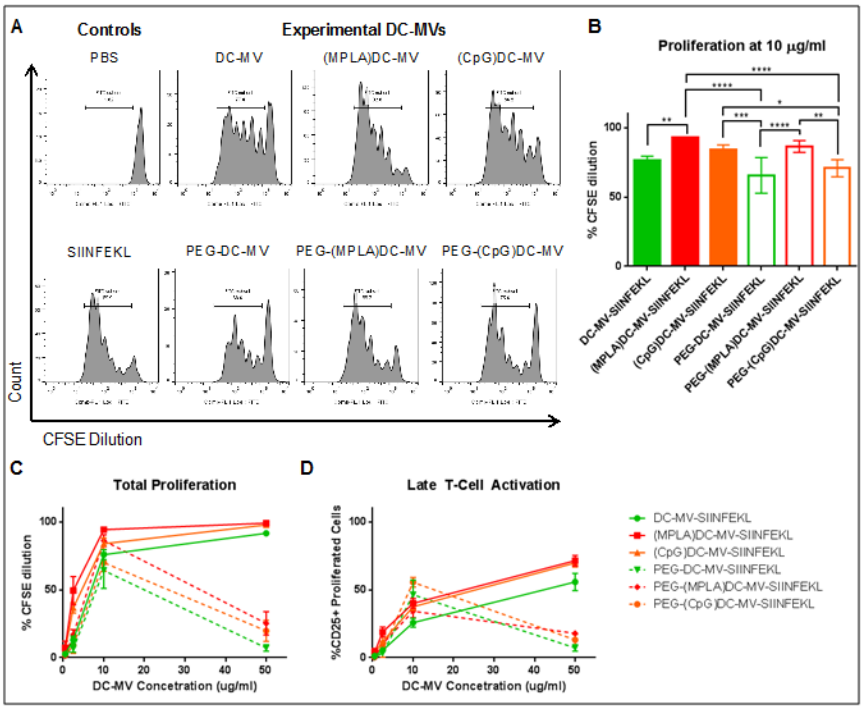

Figure 2. DC-MVs indirectly promote T cell activation and proliferation *in vitro*.

A-B. Representative histograms and summary of OT-I T cell proliferation in the presence of BMDCs and SIINFEKL-loaded DC-MVs at 10 μg/ml protein concentration of DC-MVs. **C**. Proliferation of OT-I T cells seeded with SIINFEKL-loaded DC-MVs at various concentrations. **D.** Activation was measured as the fraction of CD25-positive T cells following stimulation. Mean ± SD are shown. Statistical analysis was performed using two-way ANOVA comparison with Tukey's multiple comparison test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

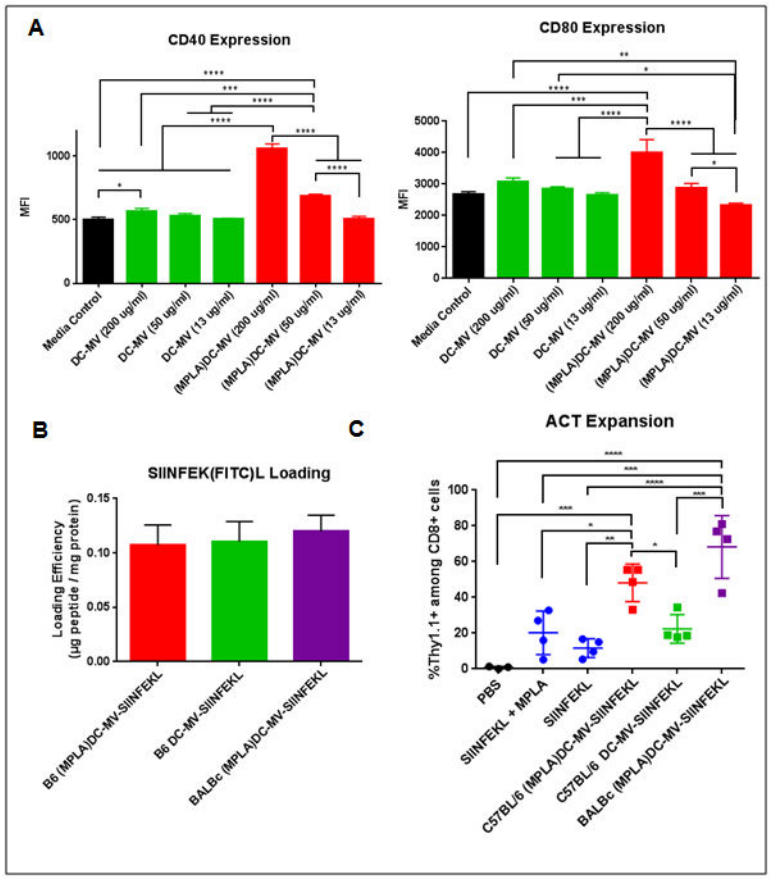

Figure 3. DC-MVs promote expansion of adoptively transferred T cells *in vivo*.

A. BMDCs were cultured *in vitro* with DC-MV formulations, and the expression of maturation markers was determined by flow cytometry. **B**. Loading of FITC-labeled SIINFEK(FITC)L peptide on various DC-MV formulations was determined using a microplate-based quantification. **C.** Congenic Thy1.2+ C57BL/6 mice were adoptively transferred with OT-I CD8α+Thy1.1+ T cells on day 0, immunized with various DC-MV formulations on day 1, and analyzed for the frequency of Thy1.1+ T cells on day 6. Mean ± SD are shown. Statistical analysis was performed using two-way ANOVA comparison with Tukey's multiple comparison test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).