Potentiation of T Cell Stimulatory Activity by Chemical Fixation of a Weak Peptide-MHC Complex

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The stability of peptide-MHC complex (pMHC) is an important factor to shape the fate of peptide-specific T cell immune response, but how it influences on T cell activation process is poorly understood. To better understand that, we investigated various T cell activation events driven by Ld MHCI loaded with graded concentrations of P2Ca and QL9 peptides, respectively, with 2C TCR Tg T cells; the binding strength of P2Ca for Ld is measurably weaker than that of QL9, but either peptides in the context of Ld interact with 2C TCR with a similar strength. When their concentrations required for early T cell activation events, which occur within several minutes to an hour, were concerned, EC50s of QL9 were about 100 folds lower than those of P2Ca, which was expected from their association constants for Ld. When EC50s for late activation events, which takes over several hours to occur, were concerned, the differences grew even larger (> 300 folds), suggesting that, due to weak binding, Ld/P2Ca dissociate from each other more easily to lose its antigenicity in a short time. Accordingly, fixation of Ld/P2Ca with paraformaldehyde resulted in a significant improvement in its immunogenicity. These results imply that binding strength of a peptide for a MHC is a critical factor to determine the duration of pMHC-mediated T cell activation and thus the attainment of productive T cell activation. It is also suggested that paraformaldehyde fixation should be an effective tool to ameliorate the immunogenicity of pMHC with a poor stability.

Keywords: chemical fixation, paraformaldehyde, peptide-MHC complex, T cell activation, T cell receptor

INTRODUCTION

Biochemical properties of a peptide to form a complex with a class I MHC (MHCI) dictate the nature of antigen-specific CD8+ T cell immune responses (Germain and Stefanova, 1999; Rudolph et al., 2006). The strength of interaction between a MHCI/peptide complex (pMHCI) and its cognate T cell receptor (TCR) is mainly determined by the amino acid sequence of the peptide loaded to MHCI (Zhu et al., 2013). When the binding strength is above the threshold, the pMHCI-TCR interaction drives full T cell activation leading to multiple rounds of cell division and development of effector functions. When it is below the threshold, the pMHCI-TCR interaction tends to induce only partial activation of T cells resulting in T cell death or anergy (unresponsiveness). Peptides with the latter property are called weak agonists or antagonists (Madrenas, 1999).

The strength of interaction between a processed peptide and a specific MHCI has also critical effect on the antigen-specific T cell immune response (Edwards and Evavold, 2013; Levitsky et al., 1996; Nelson et al., 1994). The strength of the interaction is thought to be a determining factor for...
the longevity of a specific pMHCI as well as for the propensity of pMHCI formation. Given that, the strength of a peptide-MHC interaction must have a close correlation with the density (number) of a given pMHCI on the surface of antigen-presenting cells (APCs), which is a key parameter to determine the magnitude of an antigen-specific T cell immune response (Busch and Pamer, 1998; Harndahl et al., 2012; van der Burg et al., 1996). For such a reason, many attempts have been made to enhance antigen-specific T cell immune responses by improving the properties of the peptides with modification of their amino acid sequences. Yet, it is still a task to garner such a goal without altering the specificity of TCR recognition (Khikhlo et al., 1993: 1995; Parker et al., 1992; Watson et al., 2012).

A CD8+ mouse T cell clone (H-2d) to expresses a MHCI-restricted αβ TCR designated as 2C was established in a study for allogeneic T cell immune responses (Kranz et al., 1984). 2C TCR interacts with L$^b$ MHCI when it is loaded with a specific peptide. P2Ca (LSPFPFDL) is a natural peptide identified to interact with 2C TCR in the context of L$^b$. QL9 (QLSPFPFDL), a synthetic analog of P2Ca, also interacts with 2C TCR when loaded to L$^b$. 2C TCR also interacts with an endogenous MHCI (k$^b$) when it is loaded with a peptide like dE8V (EQYKFYSV). dE8V is known to be a self-peptide involved in the positive selection of 2C TCR-expressing T cells in the thymus (Speir et al., 1998).

Studies using the iodinated P2Ca and QL9 have shown that the association constant (Ka) of P2Ca for L$^b$ is approximately 150 times higher than that of QL9 (Schieler et al., 1996; Sykulev et al., 1994a; 1994b). In line, the half maximal concentration of P2Ca (EC$_{50}$) for the expression of L$^b$ in a TAP-deficient cell line is reportedly 130 times greater than that of QL9 (Hornell et al., 2001; Schieler et al., 1996). Studies have been also carried out to measure the binding strengths of 2C TCR for its cognate antigens (i.e., L$^b$/P2Ca and L$^b$/QL9). One study using MHCI-restricted 2C T cell clone along with iodinated soluble L$^b$/peptide complexes has shown that Ka for 2C TCR-L$^b$/P2Ca interaction is about 5-10 times higher than that for 2C TCR-L$^b$/QL9 interaction. Meanwhile, an independent study using surface plasmon resonance technique (SPR) with soluble recombinant 2C TCR and the pMHCI complexes has shown that the equilibrium dissociation constant (Kd) for 2C TCR-L$^b$/P2Ca interaction (3.3 μM) is rather discernibly lower than that for 2C TCR-L$^b$/QL9 interaction (3.9 μM) (Garzia et al., 1997).

Potencies of P2Ca and QL9 for induction of 2C T cell immune functions have been also examined in several studies. In accordance with the higher binding affinity of QL9 for L$^b$, it has been shown that QL9 can perform at lower concentrations than P2Ca. The magnitudes of the difference in the effective concentrations of QL9 and P2Ca are, however, found to vary significantly depending on types of immune responses investigated. When the concentrations of P2Ca and QL9 loaded to target cells for sensitization of the 2C T cell clone are concerned, one study has shown that the EC$_{50}$ of P2Ca is about 75 folds higher than that of QL9 (Sykulev et al., 1994b). When the concentrations of the same peptides loaded to APCs for production by a 2C TCR-expressing T cell hybridoma line of IL-2 are concerned, the difference has been found even greater (over 2000 folds) (Holler et al., 2001). A study using purified CD8+ 2C TCR transgenic (Tg) T cells along with artificial APCs expressing L$^d$, B7-1 and ICAM-1 has also shown that QL9 is nearly 2000 times more potent than P2Ca in activating the T cells to undergo multiple rounds of cell divisions (Cai et al., 1996).

S2 Drosophila (Dros) cells engineered to express various immunomolecules of interest (e.g., MHCI, B7-1, ICAM-1) have been used as artificial antigen presenting cells (aAPCs) (Cai et al., 1996). In addition, nano-sized membrane vesicles either released naturally from Dros APCs or prepared from their plasma membrane after homogenization of the cells have been also found useful in investigation of diverse mechanisms of T cell activation (Hwang et al., 2003; Kim et al., 2009b; 2009c). Studies have shown that the nano-sized vesicles expressing L$^d$, B7-1 and ICAM-1, when loaded with a specific peptide (e.g., QL9), make contact with 2C TCR Tg T cells to induce intracellular signals for cell cycle progression and development of effector functions (Hwang et al., 2003). The plasma membrane-derived vesicles (pMVs), in particular, have been used in various experiments to investigate key cellular and molecular mechanisms for T cell activation: e.g., TCR-mediated integrin (LFA-1) activation (Kim et al., 2009c), LFA-1-dependent Ca$^{2+}$ entry and polymerization of F-actin, etc (Kim et al., 2009b).

In this study, Dros pMVs expressing L$^d$, B7-1 and ICAM-1 were employed to probe biological (immunological) potencies of QL9 and P2Ca for activation of 2C TCR Tg T cells. The feature of pMVs that they can be easily accommodated to various experimental settings and instrumentations made it possible to examine their potencies in induction of various cellular and molecular processes preceding to and requisite for T cell proliferation.

**MATERIALS AND METHODS**

**Mice**

2C TCR Tg mice originally generated by Dr. Loh were bred and maintained in Animal Resources Center in The Scripps Research Institute (USA) and in Central Animal Resource Center in The Chungnam National University (Korea). CD28 knockout (KO) and CD18 (LFA-1) KO mice were purchased from The Jackson Laboratory (Bar Harbor, USA) to generate 2CD28$^{-}$ and 2CLFA-1$^{-}$ mice (Kim et al., 2009b; 2009c). C57BL/6 mice were purchased from The Jackson Laboratory and Samtako Inc (Korea).

**Cell lines, mAbs and other agents**

Dros APCs expressing L$^d$ MHCI, mouse B7-1 and mouse ICAM-1 were cultured in Schneider's medium (Thermo Fisher Scientific, USA) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA). Copper sulfate (1 mM) was added to the culture to induce expression of mouse immunomolecules of interest (e.g., MHCI, B7-1, ICAM-1, LFA-1, ICAM-1), when loaded with a specific peptide (e.g., QL9), make contact with 2C TCR Tg T cells to induce intracellular signals for cell cycle progression and development of effector functions (Hwang et al., 2003). The plasma membrane-derived vesicles (pMVs), in particular, have been used in various experiments to investigate key cellular and molecular mechanisms for T cell activation: e.g., TCR-mediated integrin (LFA-1) activation (Kim et al., 2009c), LFA-1-dependent Ca$^{2+}$ entry and polymerization of F-actin, etc (Kim et al., 2009b).

CD8$^+$ 2C Tg T cells were purified from single cell suspensions prepared from whole body lymph nodes of 2C TCR Tg mice using CD8a$^+$ T Cell Isolation Kit (Milteny Biotech, Germany) followed by panning on anti-mCD8a mAb-coated plates.
An ascites fluid containing anti-mCD8a mAb (53-6.7) was prepared and used in panning (Cai et al., 1996). Phyco-erythrin (PE)-conjugated anti-mCD80 (mB7-1) (16-10A1), anti-mCD8 (53-6.7), anti-mCD28 (37.51), anti-mCD11a (M17/4) and anti-L2 (28-14-8) mAbs were purchased from eBioscience (USA). Anti-PLC-γ1 and anti-phospho-PLC-γ1 (Tyr783) mAbs were prepared from Santa Cruz Biotechnology (USA). Anti-mlL-2 (MAb702), anti-mIFN-γ (MAb785), biotinylated anti-mlL-2 (BAF402) and biotinylated anti-mIFN-γ (BAM7811) mAbs were purchased from R&D Systems (USA). EZ-Cytox™ agent was purchased from Daelilab Service (Korea).

**Preparation of Dros pMVs**

PVMs were prepared as described using sucrose gradient ultracentrifugation (Kim et al., 2009c). Briefly, Dros APCs expressing the mouse immuno-molecules were lysed using Kontes homogenizer in a hypotonic buffer containing a cocktail of protease inhibitors. Nuclei were then removed and crude membrane vesicles in the supernatant were collected by centrifugation at 50,000 x g for 1 h. The pelleted crude vesicles were resuspended in a buffer and subjected to sucrose gradient ultracentrifugation (200,000 x g, 2 h). The plasma membrane-derived vesicles were then collected from the interface of 20/35% sucrose layers. The amount of pMVs was determined by the concentration of proteins in the pMV suspension measured with BCA protein assay reagents (Thermo Fisher, USA). The prepared pMVs were aliquoted and stored at -80°C freezer. Prior to use, pMVs were thawed out on ice and loaded with a peptide for 1 h at rt.

**Staining of F-actin and measurement of intracellular [Ca2+]**

Purified 2C T cells cultured with peptide-loaded Dros pMVs were fixed with paraformaldehyde (PFA), permeabilized and stained with FITC-conjugated phalloidin as described previously (Kim et al., 2009c). The levels of F-actin in the cells represented by the fluorescence intensity of FITC were then analyzed by flow cytometry.

Kinetic analysis of intracellular [Ca2+] of 2C T cells cultured with peptide-loaded pMVs were performed with BD LSR II flow cytometer (BD Bioscience, USA) equipped with 37°C water jacket as described previously (Kim et al., 2009b). Briefly, 2C T cells loaded with Indo-1 (Thermo Fisher Scientific, USA) were suspended in DMEM medium supplemented with 10% FBS. The T cells were run on the flow cytometer for 60 s without pMVs and then mixed with peptide-loaded pMVs. The changes in intracellular [Ca2+] of the T cells were analyzed continuously afterward for 10 min.

**Western blot analysis and ELISA**

Western blot analysis was performed as described previously (Kim et al., 2009b). Briefly, 2C T cells cultured with peptide-loaded Dros pMVs were lysed in a cell lysis buffer (50 mM Trs-HCl, 150 mM NaCl, 1% Igepal CA-630, pH 7.5). Equal amounts of the T cell lysates were then separated on SDS-PAGE and subjected to Western blot analysis.

Sandwich ELISA was performed primarily as described by Voller et al. (1978). Briefly, an ELISA plate (Thermo Fisher Scientific) was coated with anti-IL-2 or anti-IFN-γ mAb in Na-carbonate buffer (100 mM, pH 9.5) overnight at 4°C. Then, the plate was loaded sequentially with a blocking buffer (1% BSA, 0.05% Tween 20, 1X PBS), culture supernatants, biotinylated anti-IL-2 or anti-IFN-γ mAb and horse radish peroxidase (HRP)-conjugated streptavidin. After loading of each reagent, the plate was incubated for 30 min or 1 h at rt followed by thorough wash. After final wash, TMB ELISA (Sigma-Aldrich) substrate was added. Color developed by HRP-TMB reaction was measured.

**PMV binding assay**

PMV binding assay was performed as described before (Hwang et al., 2003). Briefly, purified 2C Tg T cells (4-5 x 10^6) in 100 μl of DMEM medium supplemented with 10% FBS were cultured with 5 μg of peptide-loaded LB87-1ICAM-1 pMVs in round bottom 96 well plate for 1-2 h at 37°C. The T cells were then spun down and resuspended in a FACS buffer (1X PBS, 2.5% horse serum, 1% BSA) containing PE-conjugated anti-CD80 (B7-1) mAb plus APC-conjugated anti-CD8 mAb. After 30 min incubation on ice, the T cells were washed once with the FACS buffer and analyzed by flow cytometry.

**Paraformaldehyde fixation of Dros pMVs**

PFA-fixed pMVs were prepared as follows. Purified pMVs were loaded with a specific peptide for 1 h at rt. Peptide-loaded pMVs were mixed with an equal volume of freshly prepared 4% PFA in 1X PBS and incubated for 1 h at rt on a rotating wheel. Fixed pMVs were then dialyzed extensively with 1X PBS to remove PFA. The fixed pMVs were then filter-sterilized with 0.4 μm disc filter. The amount of fixed pMVs was determined by the concentration of proteins in the pMV solution.

**T cell proliferation assay**

One hundred microliter of purified 2C Tg T cells (5 x 10^5) in RPMI1640 medium containing 10% FBS, glutamine, HEPES (10 mM, pH 7.0), and 2-mercaptoethanol (50 μM) were mixed with 50 μl (200 μg/ml) of peptide-loaded natural or fixed pMVs in a 96 well plate and cultured in 37°C humidified CO2 incubator for two to three days. When [3H] thymidine was used to measure the level of T cell proliferation, [3H] thymidine was added to the culture at 1 μCi/ml 8 h before cell harvest (Cai et al., 1996). When CFSE was used to measure the level of T cell proliferation, the T cells were loaded with CFSE before culture as described previously (Quah et al., 2007). And, the level of T cell proliferation was determined by the extent of CFSE dilution.

**RESULTS**

**Efficacies of P2Ca and QL9 for TCR-directed actin polymerization**

An earlier study using purified CD8+ 2C TCR Tg T cells and QL9-loaded L87-1ICAM-1 Dros pMVs has shown that the level of filamentous (F) actin in the T cells increases immediately upon culture with the pMVs to reach a steady-state within 5 min (Hwang et al., 2003; Kumari et al., 2014). The same study also has shown that the TCR-pMHC interaction is
necessary and sufficient for the increase of F-actin. In light of those findings, efficacies of P2Ca and QL9 were compared for induction of actin polymerization.

A rapid increase of F-actin was also observed when 2C Tg T cells were cultured with the P2Ca-loaded pMVs (Fig. 1). Expectedly, however, P2Ca had to be loaded to the pMVs at higher concentrations than QL9 to attain comparable levels of actin polymerization. The EC50 of P2Ca (0.29 μM) was found to be about 60 fold higher than that of QL9 (0.005 μM). Nonetheless, the difference in the maximal levels of actin polymerization elicited by the pMVs loaded with the respective peptides at the saturated concentrations appeared marginal, if any.

**Efficacies of P2Ca and QL9 for extracellular calcium entry**

Increase in the concentration of cytosolic Ca\(^{2+}\) via the entry of extracellular Ca\(^{2+}\) is a hallmark of T cell activation (Hogan et al., 2010). An earlier study by Kim et al. using QL9-loaded L\(^{\beta}B7-1\)ICAM-1 pMVs and kinetic flow cytometry analyses has shown that culture of 2C Tg T cells with the pMVs brought about a progressive increase in cytosolic [Ca\(^{2+}\)] (Kim et al., 2009c). The same study has also shown that both 2C TCR-

pMHC and LFA-1-ICAM-1 interactions played indispensable roles in the calcium signaling. Efficacies of P2Ca and QL9 for induction of extracellular Ca\(^{2+}\) entry were examined in use of the kinetic Ca\(^{2+}\) assay. P2Ca-loaded L\(^{\beta}B7-1\)ICAM-1 pMVs brought about the entry of extracellular Ca\(^{2+}\) as well. As shown previously in the experiment using the QL9-loaded pMVs, the increase in the intracellular [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_i\)) during culture with the P2Ca-loaded pMVs also occurred progressively over the period of time before reaching the plateau (Fig. 2A). Expectedly, P2Ca had to be loaded to the pMVs at higher concentrations than QL9 to trigger comparable levels of Ca\(^{2+}\) entry. The EC50 of P2Ca (0.6 μM), the concentration of P2Ca to induce a half maximal [Ca\(^{2+}\)]\(_i\) increase, turned out about 90 fold higher than that of QL9 (0.007 μM).

**Efficacies of P2Ca and QL9 for activation of PLC-γ**

Phospholipase C-γ (PLC-γ) plays a central role in TCR-mediated intracellular signaling processes. Phosphorylation of PLC-γ1 at Tyr783, which promptly follows TCR triggering, is requisite for its signaling function (Kim et al., 2009c; Rhee 2001). Phosphorylation of PLC-γ1 at Tyr783 was observed soon after culture of 2C Tg T cells with L\(^{\beta}B7-1\)ICAM-1 pMVs loaded not only with QL9 but also with P2Ca (Fig. 2B). As

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**Fig. 1. Efficacies of QL9 and P2Ca peptides for 2C T cell actin polymerization by L\(^{\beta}B7-1\)ICAM-1 Dros pMVs.** Highly purified CD8\(^+\) 2C TCR T g T cells were cultured for 15 min at 37°C with L\(^{\beta}B7-1\)ICAM-1 Dros pMVs loaded with serially diluted concentrations of QL9 (gray) or P2Ca (black), and the levels of F-actin were determined with the fluorescence intensities measured after staining with FITC-labelled Phalloidin. (B) Histograms depicting the levels of F-actin of 2C TCR T g T cells cultured with pMVs loaded with no peptide (no filling) or QL9 (0.41, 0.005 μM) or P2Ca (3.3, 0.37 μM) are shown.

**Fig. 2. Efficacies of QL9 and P2Ca peptides for calcium signaling and PLC-γ1 activation by L\(^{\beta}B7-1\)ICAM-1 Dros pMVs.** Changes in [Ca\(^{2+}\)]\(_i\) in 2C TCR T g T cells being cultured with L\(^{\beta}B7-1\)ICAM-1 pMVs loaded with graded concentrations of P2Ca or QL9 at 37°C were measured using flow cytometry and plotted. The concentrations of each peptide loaded to pMVs were as denoted. (B) Cell extracts prepared from 2C TCR T g T cells cultured with pMVs loaded with graded concentrations of P2Ca or QL9 were subjected to Western blot analyses for phosphorylated PLC-γ1 (Tyr\(^{783}\)) and total PLC-γ1, respectively.
seen in other experiments described above, P2Ca had to be loaded to the pMVs at significantly higher concentrations than QL9 to induce comparable levels of the tyrosine phosphorylation. The EC50 of P2Ca (0.31 μM), the concentration of P2Ca required for a half maximal PLC-γ1 phosphorylation, was approximately 50 fold higher than that of QL9 (0.006 μM).

**Efficacies of P2Ca and QL9 for 2C Tg T cell absorption of L8B7-1ICAM-1 pMVs**

Earlier studies have shown that when 2C Tg T cells are cultured with QL9-loaded L8B7-1ICAM-1 pMVs, they pick up the pMVs to express molecules uniquely expressed in the pMVs on the cell surface (e.g., L8, B7-1) (Hwang et al., 2003). The same studies also have shown that specific receptor-ligand interactions, i.e., 2C TCR-L8/QL9 plus LFA-1-ICAM-1 interactions, and vital intracellular signaling mechanisms (Abram and Lowell, 2009) are mandatory for the pMV absorption. In light of those findings, efficacies of P2Ca and QL9 for instigation of 2C T cell absorption of L8B7-1ICAM-1 pMVs were examined.

Purified 2C Tg T cells picked up not only QL9-loaded but also P2Ca-loaded L8B7-1ICAM-1 pMVs (Fig. 3). As in other assays described above, P2Ca had to be loaded to the pMVs at higher concentrations than QL9 to bring about comparable levels of pMV absorption. When the T cells were cultured with the pMVs for one hour, the EC50 of P2Ca (6.5 μM) turned out about 65 fold higher than that of QL9 (0.1 μM) (Figs. 3A top and 3B). The maximal levels of pMVs absorption garnered by QL9 and P2Ca peptides after culture for one hour were comparable to each other.

When the T cells were cultured with the same pMVs for two hours, the patterns of pMV absorption changed noticeably depending on the peptide loaded to the pMVs. When QL9-loaded pMVs were used, the levels of pMV absorption increased further with a marginal change in the EC50 (about 0.08 μM). In contrast, when P2Ca-loaded pMVs were used, such increases were not found. Instead, the absorption levels were kept at the levels similar to those measured after culture for 1 h (Figs. 3A bottom and 3B). The maximal level of 2C T cell absorption of P2Ca-loaded pMVs was thus significantly lower than that of QL9-loaded pMVs. As expected from the previous studies, 2C T cell absorption of P2Ca-loaded pMVs was also strictly dependent on LFA-1-ICAM-1 interaction (Fig. 3B).

**Efficacies of P2Ca and QL9 for proliferation of 2C Tg T cells by L8B7-1ICAM-1 pMVs**

Purified 2C Tg T cells are fully activated to undergo multiple rounds of cell division and to produce effector cytokines such as IFN-γ when cultured with QL9-loaded L8B7-1ICAM-1 pMVs (Kim et al., 2009a). Potencies of QL9 and P2Ca peptides for proliferation of 2C Tg T cells by the pMVs were examined. As expected from the previous studies, a robust proliferation of purified 2C Tg T cells was observed when they were cultured with QL9-loaded L8B7-1ICAM-1 pMVs. A maximal level of [H3] thymidine incorporation was obtained when they were cultured with the pMVs loaded with the peptide at 3.3 μM; the EC50 was determined at 0.8 μM (Fig. 4A). Meanwhile, when the P2Ca-loaded pMVs were used in the culture, a significant level of [H3] thymidine incorporation was hardly observed until the T cells were cultured with
pMVs loaded with the peptide at 30 µM. While a further increase in [³H]-incorporation was observed when they were cultured with the pMVs loaded with 90 µM of P2Ca, its level was only comparable to that obtained with 0.37 µM QL9. Those results indicated that QL9 peptide was almost 300 times more potent than P2Ca in stimulating 2C T cells to undergo multiple cell divisions.

T cells cultured in vitro tend to undergo apoptotic cell death unless proper stimuli, e.g., cognate TCR-pMHC plus costimulatory receptor-ligand interactions, are provided. Conversely, T cells activated by TCR-pMHC and costimulatory receptor-ligand interactions survive to form blasts (Friedl and Gunzer, 2001). Survival and blast formation of 2C Tg T cells cultured with QL9- and P2Ca-loaded LdB7-1ICAM-1 pMVs, respectively, were examined. Here, pMVs were loaded with 1.0 µM of QL9 and 30 µM of P2Ca, respectively. When purified 2C Tg T cells were cultured with the pMVs loaded with no peptide, most T cells died within 24 h, and the blast formation was hardly observed (Fig. 4B). When the same T cells were cultured with pMVs loaded with QL9 for one day, a majority of the T cells survived to have formed blasts. The blast formation continued to progress so that the T cells cultured for two days revealed further increase in the forward scatter (FSC). When the same T cells were cultured with P2Ca-loaded pMVs for one day, a majority of the T cells survived to have formed blasts as well. In a striking contrast to the T cells cultured with QL9-loaded pMVs, however, the T cells cultured with P2Ca-loaded pMVs for two days showed no further changes in the forward and side scatter patterns compared to the T cells cultured for one day. When examined after three days, regardless of the peptide loaded to the pMVs, they showed a significant decrease in the forward and side scatters and in the ratios of live cells.

2C Tg T cells cultured with pMVs loaded with neither peptide showed little sign of CFSE dilution (Fig. 4C). When the Tg T cells were cultured with QL9-loaded pMVs, a prominent decrease in the intensity of CFSE was observed after two days. When the T cells were cultured with P2Ca-loaded pMVs, a lower but measurable level of CFSE was detected after one day. In contrast, no significant production of IFN-γ was detected throughout the culture.

No measurable production of either IL-2 or IFN-γ was detected, when the Tg T cells were cultured with the pMVs without a peptide loaded (Fig. 4D). When the same T cells were cultured with QL9-loaded pMVs one day, a significant level of IL-2 was detected in the culture supernatant, but its level decreased to an unmeasurable level the next day. Moderate levels of IFN-γ were also detected in the same culture supernatants obtained both after one and two days of culture. When the T cells were cultured with P2Ca-loaded pMVs, a lower but measurable level of IL-2 was detected after one day. In contrast, no significant production of IFN-γ was detected throughout the culture.
Enhancement of T cell stimulatory potency of P2Ca-loaded pMVs by paraformaldehyde fixation

It was somewhat puzzling that QL9 revealed over 300 times higher potency than P2Ca in stimulating 2C Tg T cells to proliferate while, in all other experiments described above, the former consistently showed only 50 to 90 times higher potencies than the latter. MHCI easily loses its immunological activity when present at 37°C without a peptide loaded (Stern and Wiley, 1992). Based on those, we speculated that the structural stability of Ld/P2Ca was low compared to that of Ld/QL9 and, thus, Ld might dissociate from P2Ca more frequently to gradually lose its conformation and the immunological activity over a period of time. In an attempt to test the hypothesis, LdB7-1ICAM-1 pMVs loaded with either QL9 or P2Ca peptide were treated with paraformaldehyde (PFA) (Eltoum et al., 2001), a widely used chemical fixative, whose treatment likely caused covalent cross-linkage between Ld and the peptide to keep the complex intact for a longer period of time.

Indeed, PFA-treatment enhanced T cell stimulatory activity of p2Ca-loaded pMVs considerably. Thus, when 2C Tg T cells were cultured with L8B7-1ICAM-1 pMVs loaded with no peptide or p2Ca (30 μM), or QL9 (1 μM) and treated with PFA. Purified 2C TCR Tg T cells labeled with CFSE were cultured with the fixed pMVs for 3 days. Two dimensional plots for FSC and SSC of 2C T cells cultured with respective pMVs for 1, 2 and 3 days are shown. (C) Histograms depicting the fluorescence intensities of CFSE of 2C T cells cultured as in (B) are shown. # denotes that the scale of Y axis of the plot is twice larger than that of others. (D) Production of IL-2 and IFN-γ by 2C TCR Tg T cells cultured as in (B) were analyzed after 1 (black bar) and 2 (white bar) days of culture by measuring the concentrations of those cytokines in the culture supernatants.
CFSE was observed in most 2C T cells cultured with those pMVs for two days (Fig. 5C). When the Tg T cells cultured with the QL9-loaded fixed pMVs for two days were analyzed, no significant differences were observed compared to the cells cultured with the QL9-loaded natural pMVs. Thus, blast formation continued to progress for two days and the ratio of live cells also increased. When the cells cultured with the fixed pMVs were analyzed the third day, further decrease in the intensities of CFSE were observed in the majority of the T cells regardless of the peptides loaded to the pMVs (Figs. 5B and 5C).

2C Tg T cells cultured with the fixed pMVs, regardless of the peptides loaded, produced high levels of IL-2 within 24 h of culture (Fig. 5D). The concentrations of IL-2 in the culture supernatants decreased almost to the basal levels, however, when they were measured after two days of culture, suggesting a vigorous consumption of the cytokine by the T cells during proliferation stage. Only moderate levels of IFN-γ were detected when they were measured after one day, but the concentrations increased considerably afterward. Of note, 2C Tg T cells cultured with the P2Ca-loaded fixed pMVs for two days, like the T cells cultured with the QL9-loaded fixed pMVs, produced a high level of IFN-γ.

**Rapid loss of T cell stimulatory activity of p2Ca-loaded natural pMVs and preservation of the activity by PFA-fixation**

Next, we investigated how quickly P2Ca-loaded natural pMVs lost their T cell stimulatory activity at 37°C. For that investigation, P2Ca-loaded natural pMVs were pre-incubated at 37°C for different periods of time before culture with purified 2C Tg T cells. As shown in Fig. 6, it was found that natural
pMVs loaded with 30 μM P2CA lost most of their activity within 12 h of pre-incubation at 37℃. When they were pre-incubated for 24 h, almost complete loss of the activity was observed. Diminution of the activity was clearly detected even after 6 hrs of pre-incubation. In contrast, the activity of P2Ca-loaded fixed pMVs was sustained for much longer period of time. Thus, the loss of the activity of the fixed pMVs was found only marginal even after 24 h of pre-incubation at 37℃.

Different from P2Ca-loaded natural pMVs, the same pMVs loaded with 1 μM QL9 could keep the activity much longer period of time. Thus, when the T cells were cultured with QL9-loaded natural pMVs which had been pre-incubated for 24 h at 37℃, they were able to undergo progressive blast formation for 48 h. Accordingly, it was also shown that PFA-treatment had little effect on the duration of T cell stimulatory activity of QL9-loaded pMVs.

Roles of co-stimulatory receptor-ligand interactions in T cell proliferation by fixed pMVs

CD28-B7-1 and LFA-1-ICAM-1 interactions are indispensable for proliferation of 2C Tg T cells by QL9-loaded natural L^B7-1ICAM-1 pMVs (Bachmann et al., 1997) (Fig. 7A). In order to examine the importance of those interactions in proliferation of the Tg T cells by the fixed pMVs, 2C Tg T cells deficient in the expression of either CD28 or LFA-1 were used. As shown in Figs. 7C and 7D, the size of 2C CD28^−/− T cells cultured with the fixed pMVs, regardless of the peptide loaded to the pMVs, was markedly smaller than that of the wild type T cells when analyzed after one and two days of culture. Moreover, the extents of CFSE dilution measured after two days of culture showed that only a minor population of the cells had proliferated during the culture, and even the proliferated cells had undergone only one or two cell divisions. These results seemed to reiterate the unique role of

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Fig. 7. Dependence of T cell proliferation by natural or fixed L^B7-1ICAM-1 Dros pMVs on co-stimulations by CD28 and LFA-1. Wild type (WT), CD28-deficient (CD28 KO) or LFA-1-deficient (β2 KO) 2C TCR Tg T cells were cultured with either natural (A, B) or PFA-fixed (C, D) L^B7-1ICAM-1 pMVs loaded with either 1 μM QL9 (A and C) or 30 μM P2Ca (B, D) for 1 or 2 days. Two-dimensional plots (top) for FSC and SSC of T cells cultured for 1 or 2 days are shown. Histograms (bottom) depicting the fluorescence intensities of CFSE of cultured 2C T cells are shown. # denotes that the scale of Y axis of the plot is twice larger than that of others.
co-stimulatory signaling receptor(s) typified by CD28 in activation and proliferation of T cells (Rosette et al., 2001). Nevertheless, compared with the same knock-out T cells cultured with the natural pMVs, more T cells were kept alive when cultured with the fixed pMVs and the live T cells showed a clear increase in cell size judged by FSC.

In contrast, LFA-1 appeared to play a dispensable role in activation/proliferation of 2C Tg T cells by the fixed pMVs. Different from 2C.CD28 

\[ CD28 \]

Tg T cells, 2C.LFA-1 

\[ LFA-1 \]

Tg T cells showed a progressive blast formation. Thus, when examined after two days of culture, LFA-1 

\[ LFA-1 \]

and the wild type 2C Tg T cells showed little difference in the extents of blast formation. In line, when cultured with fixed L 

\[ L \]

B7-1ICAM-1 pMVs, regardless of the peptides loaded to the pMVs, most LFA-1 

\[ LFA-1 \]

Tg T cells underwent robust cell division cycles.

**DISCUSSION**

Dros pMVs have several advantages in investigating cellular and molecular mechanisms for T cell activation. Firstly, due to the nanometric nature of pMVs, T cells can be easily separated from pMVs after culture, which makes prompt analysis of T cells possible without delay for separation of T cells from APCs. Secondly, as pMVs can interact with T cells in suspension, kinetic analyses using flow cytometry of T cells interacting with pMVs become possible. Thirdly, as pMVs express physiological ligands, results obtained from T cells stimulated with pMVs are likely to hold better physiological relevance compared those obtained from T cells stimulated with receptor-specific mAbs or chemicals.

When concerned are actin polymerization, increase in [Ca\(^{2+}\)], activation of PLC-\[\gamma\] and activation of integrin (LFA-1), all of which take place within a few minutes to an hour after beginning of culture with QL9- or P2Ca-loaded L 

\[ L \]

B7-1ICAM-1 pMVs, the differences in the EC\(_{50}\) of QL9 and P2Ca for induction of respective T cell activation events were consistently lower than 100 fold (50-90 fold difference). Given the reports that QL9 has about 150 fold lower Ka for L \[ L \] 

than P2Ca (Schlueter et al., 1996; Sykulev et al., 1994a; 1994b) and L \[ L \] 

/P2Ca has the slightly lower Kd for 2C TCR than L/QL9 (Garcia et al., 1997), it was expected that P2Ca had to be loaded to pMVs at approximately 100-150 fold higher concentration than QL9 to have comparable read-outs in those assays. While the differences in the EC\(_{50}\) (50-90 fold) obtained from the assays listed above appeared to largely reflect the differences in their biochemical properties, they were about 1.5-2 fold lower than the expected, suggesting that P2Ca can perform better than expected at least at the beginning of the culture. It is of interest that the results obtained from the assays mentioned here is highly consistent with the result reported by others earlier that the concentrations of QL9 loaded to the target cells for sensitization of effector 2C T cell clone is about 75 fold lower than that of P2Ca (Sykulev et al., 1994b).

When the production of IL-2 and IFN-\[\gamma\] by 2C T cells and their proliferation during culture with the same pMVs are concerned, the differences in their potencies turned out even greater. Thus, QL9 showed almost over 300 fold higher potencies than P2Ca. While still debating, it is well accepted that, different from early activation events like actin polymerization, Ca\(^{2+}\) mobilization and integrin activation, full activation of T cells leading to production of effector cytokines and multiple cell divisions requires prolonged TCR-pMHC interaction.

A study has shown that at least 20 h of TCR-pMHC engagement is necessary for full activation of T cells by a specific MHC-peptide complex (Rosette et al., 2001). It is also known that MHCI easily loses its conformation at 37°C when expressed on the cell surface without a loaded peptide (Stern and Wiley, 1992). On the basis of those information, we hypothesized that L/QP2Ca complex ought to be less stable than L/QL9 and thus might dissociate more easily (frequently) from each other. We also postulated that L free from P2Ca might gradually lose its natural conformation and intrinsic immunological activity over the period of time at 37°C. It is not clear how quickly L/P2Ca loses its antigenicity. But the results from the pMV absorption assay indicate that it begins to lose its activity within a couple of hours of culture with T cells at 37°C.

PFA has been routinely used as a fixative to preserve cellular structures of biospecimens (Eltoum et al., 2001). It reacts with amino groups in proteins to form covalent linkages among them. Such cross-linking chain reactions results in fixation of the cellular structures. We attempted to stabilize L/P2Ca complexes by exploiting the chemical property of PFA. That is, it was pondered that treatment of L 

\[ L \]

B7-1ICAM-1 pMVs loaded with P2Ca and QL9, respectively, with PFA resulted in covalent bond formation between L \[ L \]  

and the peptide to stabilize the complexes. A criticism associated with the chemical fixation of pMHCI was that the chemical treatment might cause alteration in the molecular structure of the complex to change its antigenic properties. Yet, the results that the QL9-loaded fixed (PFA-treated) pMVs stimulated 2C Tg T cells no less effectively than QL9-loaded natural pMVs argued that the structural change caused by the PFA treatment was not significant, if any, enough to alter its antigenic property.

As expected, PFA treatment of L 

\[ L \]

B7-1ICAM-1 pMVs loaded with P2Ca greatly enhanced their potency for 2C T cell activation. Thus, different from the P2Ca (30 \[\mu\]M)-loaded natural pMVs, the same pMVs treated with PFA became capable of stimulating the T cells to produce high levels of IL-2 and IFN-\[\gamma\] and to undergo multiple rounds of cell division cycles. Compared to the P2Ca-loaded pMVs, the effect of PFA fixation on the potency of the QL9-loaded pMVs appeared less significant. We assumed that that was likely due to a superior stability of L/QL9 complex. That is, it was conceived that the complex is already stable enough to continuously stimulate the T cells for a sufficient period of time to have the T cells develop effector functions and undergo proliferation.

Nevertheless, the amelioration of the T cell stimulatory activity of QL9-loaded pMVs following PFA fixation was noticed clearly in the experiments using 2C Tg T cells deficient in LFA-1 (B2 integrin) expression. Reflecting the importance of costimulatory and adhesion receptors in T cells activation, both 2C.CD28 

\[ CD28 \]

and 2C.LFA-1 

\[ LFA-1 \]

cells failed to undergo full blast formation and proliferation when cultured with natural L 

\[ L \]

B7-1ICAM-1 pMVs loaded with either QL9 or P2Ca. The
similar results were also obtained when 2C.CD28-/- Tg T cells were cultured with the PFA-fixed pMVs loaded with either of those peptides, reassuring the unique role of CD28 in T cell activation. Previous studies by others have shown that the signaling via CD28 is critical for survival of activated T cells and production of IL-2 by stabilizing its mRNAs (Alegre et al., 2001).

In contrast to 2C.CD28-/- T cells, 2C.LFA-1-/- Tg T cells underwent full activation and strong proliferation when cultured with the fixed pMVs loaded with not only QL9 but also P2Ca. While the precise mechanism underlying these results are yet to be understood, they seem to suggest that the role of LFA-1 in T cell activation either as an adhesion or as a signaling receptor (Shimizu, 2003) become dispensable when the signaling through TCR is sustained for an extended period of time. Roles of LFA-1 in T cell activation have been studied extensively. According to a study by Bachmann et al. (1997), LFA-1 plays indispensable role when T cells are activated by only a limited amount of pMHCs by reducing the threshold for the level of antigens required for full T cell activation. The same study also has shown that LFA-1 plays only a dispensable role when T cells are activated by an excess amount of pMHCs. Our results imply that a persistent presence of a low amount of antigens may also discount the importance of LFA-1 in T cell activation. Further studies must be carried out to understand how persistent TCR-pMHC engagements allow productive T cell activation to take place without LFA-1. Here, it seems worth noting that a study by Viola and Lanzavecchia (1996) has shown that the consequence of T cell activation is determined by a number of triggered TCRs. Thus, it seems possible that the fixed pMVs, which can be present in the culture for an extended period of time with the intact conformation and antigenicity, can continue to trigger TCR to reach the threshold level for T cell activation without LFA-1.

In accordance with our results, a recent study by Liu et al. (2014) has shown that PFA-treatment of DCs to express an immune complex formed by MHCI-like molecule Qa1 and HSP60-derived peptide (Hsp60sp) results in amelioration of the immunological property of DCs in a mouse model of multiple sclerosis. They have also indicated that potentiation of the immunological activity of Qa1-Hsp60sp complex results from stabilization of the complex by PFA (Liu et al., 2014). While the results by Liu et al. along with our own results suggest that the PFA-fixation method should be used more broadly for amelioration of immunological properties of pMHCs, further studies with a variety of pMHCs must be done to draw such a conclusion.

Advances in DNA sequencing technology (Buermans and den Dunnen, 2014) make it possible to decode the entire human genome sequences in a matter of days with a reasonable cost. Such a development also makes it possible to identify genetic changes made to the protein coding sequences in the course of tumorigenesis and thus potential tumor-associated antigens (TAAs) (Butterfield, 2015) in individual tumors. Indeed, the inventory of TAAs has been growing steadily ever since. But, the clinical application of those TAAs to tumor immunotherapy has been limited as a variety of factors related with TAAs have effect on the clinical use, which include the presence of required sequence elements for antigen processing by proteasome (Kloetzel, 2001), the binding affinities of the processed peptides for partnering MHCs and the stabilities of the resulting pMHCs. The stability of pMHCs formed with tumor-specific peptides is thought to be a particularly important factor for the clinical application of TAAs as modification of peptide sequence to improve the stability without altering antigenic specificity is a highly challenging task. Here, we propose that PFA fixation can be a viable method to improve the stability of pMHCs and can be useful in development of cell free tumor T cell vaccines (Robbins and Morelli, 2014; Viaud et al., 2010).

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