LFA-1-dependent Ca\(^{2+}\) Entry following Suboptimal T Cell Receptor Triggering Proceeds without Mobilization of Intracellular Ca\(^{2+}\) 

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A surge in cytosolic calcium ion concentration by entry of extracellular Ca\(^{2+}\) is a hallmark of T cell activation. According to store-operated Ca\(^{2+}\) entry mechanism, the Ca\(^{2+}\) entry is preceded by activation of phospholipase C-\(\gamma\) (PLC-\(\gamma\)) and the consequent mobilization of intracellular Ca\(^{2+}\). Using membrane vesicles expressing the mouse class I major histocompatibility complex, i.e. Ld plus costimulatory ligands, i.e. B7-1 and intercellular adhesion molecule-1 along with 2C T cell receptor transgenic T cells, we investigated the roles of CD28 and LFA-1 (lymphocyte function-associated antigen-1) in the activation of PLC-\(\gamma\) and Ca\(^{2+}\) signaling. Both CD28 and LFA-1 made significant and comparable contributions to the activation of PLC-\(\gamma\) as gauged by the level of its phosphorylation at tyrosine 783. In contrast, their roles in Ca\(^{2+}\) signaling were quite distinct so that LFA-1/intercellular adhesion molecule-1 interaction exerted a determining role, whereas CD28/B7-1 interaction played only a minimal role. In particular, when the T cells were activated by suboptimal T cell receptor stimulation, LFA-1 played an indispensable role in the Ca\(^{2+}\) signaling. Further experiments using Ca\(^{2+}\)-free medium demonstrated that the entry of extracellular Ca\(^{2+}\) was not always accompanied by mobilization of intracellular Ca\(^{2+}\). Thus, intracellular Ca\(^{2+}\) mobilization was hardly detected under the condition that LFA-1 played the indispensable role in the entry of extracellular Ca\(^{2+}\), while a distinct level of intracellular Ca\(^{2+}\) mobilization was readily detected under the condition that LFA-1 played only the supporting role. These results ensure the unique role of LFA-1 in T cell Ca\(^{2+}\) signaling and reveal that LFA-1-dependent Ca\(^{2+}\) entry proceeds via a mechanism separate from store-operated Ca\(^{2+}\) entry.

The T cell encounter with antigen-presenting cells (APCs)\(^2\) carrying cognate peptide in the context of major histocompatibility complex (MHC) is followed by an increase in cytosolic Ca\(^{2+}\) ion concentration ([Ca\(^{2+}\)]) by entry of extracellular Ca\(^{2+}\). The extracellular Ca\(^{2+}\) entry is key for a myriad of physiological changes leading to cell cycle progression and development of effector functions of T cells. Downstream signaling events requiring an influx of extracellular Ca\(^{2+}\) involve activation of calcineurin/NF-AT and Ras/mitogen-activated protein kinase (MAPK) signaling pathways together resulting in transcriptional activation of multiple genes, including interleukin-2 (2, 3).

A well known Ca\(^{2+}\)-signaling mechanism in T cells is store-operated Ca\(^{2+}\) entry (SOCE). According to that mechanism, phospholipase C-\(\gamma\) (PLC-\(\gamma\)) activated by T cell receptor (TCR) stimulation catalyzes hydrolysis of phosphatidylinositol 4,5-biphosphate to produce inositol 1,4,5-trisphosphate (IP\(_3\)), which binds to the IP\(_3\) receptor in the endoplasmic reticulum to induce release (mobilization) of Ca\(^{2+}\) from the endoplasmic reticulum. As a result, Ca\(^{2+}\) channel in plasma membrane opens to allow entry of extracellular Ca\(^{2+}\). Recent advances in the field entail identification of specific proteins involved in the process such as STIM-1, a Ca\(^{2+}\) sensor in endoplasmic reticulum, and ORAI-1, a Ca\(^{2+}\) channel in plasma membrane (4).

LFA-1 (lymphocyte function-associated antigen-1), an integrin composed of \(\alpha\)L (CD11a) and \(\beta\)_2 (CD18) subunits, plays multiple roles in various stages of T cell immune responses, i.e. in activation of resting T cells, migration of activated effector T cells to the site of infection, and execution of their effector functions (5, 6). LFA-1 acts as both an adhesion and a signaling molecule so that interaction of LFA-1 with its ligand, intercellular adhesion molecule-1 (ICAM-1), not only facilitates firm contact between T cell and APC but also induces intracellular signaling events (7).

The importance of LFA-1 in Ca\(^{2+}\) signaling has been identified. Earlier studies showed that engagement of LFA-1 resulted in prolonged IP\(_3\) production and the sustained increase of intracellular [Ca\(^{2+}\)] as well as stronger PLC-\(\gamma\) activation (8, 9). It was also shown that a specific motif (NPXY) in the intracellular domain of the CD18 subunit was responsible for the action of LFA-1 (10). In addition, a study by Bachmann et al. (11) showed that costimulation by LFA-1 lowered the threshold level of cognate peptide-MHC complex (pMHC) expression required for induction of extracellular Ca\(^{2+}\) entry during the T/APC interaction. It was also suggested that LFA-1 facilitated entry of extracellular Ca\(^{2+}\) by promoting the formation of immunological synapse by which production of IP\(_3\) could be amplified and stabilized (12).
LFA-1 and Calcium Signaling

We have been using nanometric membrane vesicles prepared from the purified plasma membrane fraction of Drosophila cells engineered to express L^d class I MHC plus B7-1 and ICAM-1, along with 2C TCR transgenic T cells, for studying membrane-proximal TCR signaling events leading to LFA-1 activation (13, 14). In this study, we investigated Ca^{2+} signaling of 2C T cells being cultured with the plasma membrane-derived membrane vesicles (pMVs), and we present data revealing that LFA-1-dependent Ca^{2+} entry, manifest in T cells undergoing the activation process after suboptimal TCR stimulation, proceeds via a mechanism separate from SOCE.

EXPERIMENTAL PROCEDURES

Animals—CD28^-/-^ (B6) and β2-integrin^-/-^ (LFA-1^-/-^) (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained at The Scripps Research Institute. Wild type, CD28^+^ (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained at The Scripps Research Institute. (B6) mice were bred at The Scripps Research Institute.

Peptides, Chemicals, and mAbs—QL9 (QLSPFPFDL), P2Ca (LSPPFPFDL), and P1A (LPYLGWLVF) peptides (13) were purchased from GenScript. Anti-LFA-1-dependent Ca^{2+} signaling and calcium signaling were examined by syringe biopsy using a flow cytometer equipped with a temperature control device. Changes in the concentration of intracellular Ca^{2+} were determined by FCM using Indo-1/Am (Invitrogen) for 2h to detect calcium entry.

RESULTS

Effects of CD28/B7-1 and LFA-1/ICAM-1 Interactions on Activation of PLC-γ1 and PI3K/Akt Signaling Cascades—We had shown that culture of 2C T cells with QL9 (or P2Ca) peptide-loaded L^d/B7-1/ICAM-1 Drosophila pMVs resulted in activation of membrane-proximal signaling cascades involving protein-tyrosine kinases (PTKs) and phosphoinositide 3-kinase (PI3K) exemplified by tyrosine phosphorylation of PLC-γ1 (19) and threonine phosphorylation of Akt (14, 20). In this study, we examined the contributing roles of TCR/pMHC, CD28/B7-1, and LFA-1/ICAM-1 interactions in the phosphorylation of PLC-γ1 and Akt.

Phosphorylation of Akt at Thr^{308} occurred rapidly upon culture of 2C T cells with the pMVs loaded with QL9 (1 μM) peptide. The level of the Akt phosphorylation reached near maximum within 5 min of culture and was sustained through 30 min (Fig. 1A, left). Phosphorylation of PLC-γ1 at Tyr^{783} was also detected at 5 min of the culture, but the level of the phosphorylation increased gradually as the culture time extended (Fig. 1B, left).
to the mechanism of SOCE, entry of extracellular Ca\(^{2+}\) resulting in a surge in cytosolic [Ca\(^{2+}\)] was preceded by mobilization of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores following PLC-\(\gamma\) activation in a surge in cytosolic [Ca\(^{2+}\)] (Fig. 2). The increase became evident ~2.5 min after mixing of 2C T cells with the pMVs and continued through the culture up to 12 min.

mAb blocking of CD28/B7-1 interaction slightly reduced the extent of Ca\(^{2+}\) increase (Fig. 2, left). In contrast, mAb blocking of LFA-1/ICAM-1 interaction almost completely abolished the Ca\(^{2+}\) increase. Importance of LFA-1/ICAM-1 interaction in the cytosolic Ca\(^{2+}\) increase was confirmed by experiments using 2C LFA-1\(^{-/-}\) T cells, in which no measurable Ca\(^{2+}\) increase was observed. Consistent with the result obtained with 2C T cells treated with anti-CD28 mAb, 2C CD28\(^{-/-}\) T cells showed conspicuous increase in cytosolic [Ca\(^{2+}\)] during the culture (Fig. 2).

### Lack of Intracellular Ca\(^{2+}\) Mobilization in 2C T Cells Cultured with L\(^{6}\)B7-1ICAM-1 pMVs—According to the model, CD28/B7-1 and LFA-1/ICAM-1 interactions had positive effects on the PLC-\(\gamma\) and Akt phosphorylation (Fig. 1, A and B, right, and supplemental Fig. S1). Thus, wild type 2C T cells treated with either anti-CD28 or anti-LFA-1 mAb and mutant 2C T cells deficient in CD28 (2C CD28\(^{-/-}\)) or LFA-1 (2C LFA-1\(^{-/-}\)) expression showed significantly reduced levels of phosphorylation of both PLC-\(\gamma\) and Akt compared with wild type 2C T cells treated with control mAb. A further decrease in levels of phosphorylation was observed when the wild type 2C T cells were co-treated with anti-CD28 plus anti-LFA-1 mAbs, and 2C CD28\(^{-/-}\) and 2C LFA-1\(^{-/-}\) T cells were treated with anti-LFA-1 and anti-CD28 mAbs, respectively.

### Increase of Cytosolic [Ca\(^{2+}\)] during Culture of 2C T Cells with the pMVs—Observation that the tyrosine phosphorylation of PLC-\(\gamma\) occurred quickly during culture of 2C T cells with the pMVs loaded with QL9 peptide prompted us to examine Ca\(^{2+}\) signaling during the culture.

Wild type 2C T cells being cultured with the QL9 (1 \(\mu\)M)-loaded L\(^{6}\)B7-1ICAM-1 pMVs showed a prominent increase in cytosolic [Ca\(^{2+}\)] (Fig. 2). The increase became evident ~2.5 min after mixing of 2C T cells with the pMVs and continued through the culture up to 12 min.

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were analyzed as in Fig. 2.

Flow cytometric analyses were performed as in Fig. 2.

2C T cells cultured with L\textsuperscript{(high)}B7-1ICAM-1 pMVs loaded with QL9 (1 \muM) peptide in \textsuperscript{45}Ca\textsuperscript{2+}-containing medium showed a rapid and progressive increase in the concentration of cytosolic \textsuperscript{45}Ca\textsuperscript{2+} (Fig. 3B, right). The [\textsuperscript{45}Ca\textsuperscript{2+}] measured after 12 min of culture with QL9-loaded L\textsuperscript{(high)}B7-1ICAM-1 pMVs was higher than that measured after culture with L\textsuperscript{(high)}B7-1ICAM-1 pMVs, but the difference was marginal. As with QL9-loaded L\textsuperscript{d} pMVs, rapid and transient increase in cytosolic [\textsuperscript{45}Ca\textsuperscript{2+}] was detected when 2C T cells were cultured with QL9-loaded L\textsuperscript{(high)}B7-1ICAM-1 pMVs in \textsuperscript{45}Ca\textsuperscript{2+}-free medium (Fig. 3B, right).

**Level of Cognate pMHC Expression Versus LFA-1 Dependence of \textsuperscript{45}Ca\textsuperscript{2+} Signaling**—Next, we examined an effect of concentration of QL9 peptide loaded to L\textsuperscript{(high)}B7-1ICAM-1 pMVs on both entry of extracellular \textsuperscript{45}Ca\textsuperscript{2+} and mobilization of intracellular \textsuperscript{45}Ca\textsuperscript{2+} observed in \textsuperscript{45}Ca\textsuperscript{2+}-containing and \textsuperscript{45}Ca\textsuperscript{2+}-free media, respectively (Fig. 4). When wild type 2C T cells were cultured with the pMVs loaded with QL9 peptide at 200 and 40 nM, respectively, both mobilization of intracellular \textsuperscript{45}Ca\textsuperscript{2+} and influx of extracellular \textsuperscript{45}Ca\textsuperscript{2+} were readily detected. When the pMVs were loaded with the peptide at 8 nM, a considerable increase in cytosolic [\textsuperscript{45}Ca\textsuperscript{2+}] was detected when the T cells were cultured in \textsuperscript{45}Ca\textsuperscript{2+}-containing medium, although no measurable change was monitored when cultured in \textsuperscript{45}Ca\textsuperscript{2+}-free medium. When the pMVs were loaded with the peptide at a further lower concentration (1.6 nM), no \textsuperscript{45}Ca\textsuperscript{2+} increase was detected in either medium (Fig. 4, left).

When 2CLFA-1\textsuperscript{1/-/-} T cells were used in the experiments (Fig. 4, right), different results were obtained. As with wild type 2C T cells, the mutant T cells being cultured with L\textsuperscript{(high)}B7-1ICAM-1 pMVs loaded with QL9 peptide at higher concentrations (200 and 40 nM, respectively) displayed both influx of extracellular \textsuperscript{45}Ca\textsuperscript{2+} and mobilization of intracellular \textsuperscript{45}Ca\textsuperscript{2+}. The pattern of \textsuperscript{45}Ca\textsuperscript{2+} increase in the mutant 2C T cells cultured in \textsuperscript{45}Ca\textsuperscript{2+}-containing medium was, however, different from that in wild type 2C T cells and rather resembled that in wild type 2C T cells cultured with QL9-loaded L\textsuperscript{d} pMVs (Fig. 3B). When the same mutant T cells were cultured with L\textsuperscript{(high)}B7-1ICAM-1 pMVs loaded with the peptide at lower concentrations (8 and 1.6 nM, respectively), they showed no \textsuperscript{45}Ca\textsuperscript{2+} increase in either medium.

**Role of PLC-\gamma 1 in LFA-1-dependent Extracellular \textsuperscript{45}Ca\textsuperscript{2+} Entry**—We also examined the involvement of key signaling molecules engaged in membrane-proximal TCR signaling, i.e. PTKs, PI3K, and PLC-\gamma 1, in LFA-1-dependent \textsuperscript{45}Ca\textsuperscript{2+} entry using pharmacological agents specifically targeting those molecules. Treatment of wild type 2C T cells with PP2, Ly294002, or U73122, which target PTKs, PI3K, and PLC-\gamma 1, respectively, before culture with L\textsuperscript{B7-1ICAM-1} pMVs loaded with QL9 (1 \muM) peptide strongly inhibited the \textsuperscript{45}Ca\textsuperscript{2+} entry (Fig. 5), indicating a critical role of those signaling molecules in the LFA-1-dependent \textsuperscript{45}Ca\textsuperscript{2+} entry. The importance of PI3K was further sup-
ported by comparable inhibition of the Ca$^{2+}$ entry by wortmannin (500 nM), another well known PI3K inhibitor. 3

**DISCUSSION**

Microvesicles prepared from plasma membrane of Drosophila APCs expressing defined mouse immunomolecules of interest (e.g. pMVs) (13, 14, 22) have several advantageous features in investigation of membrane-proximal signaling mechanisms primed by TCR stimulation. Given that physiological ligands expressed in biological membrane are used for stimulating T cells, experimental results obtained with pMVs may have superior physiological relevance compared with those obtained with receptor-specific mAbs or ligands immobilized on the surface of plastic or magnetic beads. Because of the huge difference in their sizes, separation of T cells from pMVs after culture is simple, and thus instant biochemical analysis of molecular alterations occurring during the culture becomes feasible; when intact cells are used for stimulating T cells, a complicated method is needed to separate T cells from stimulating cells. In addition, kinetic flow cytometric analysis of physiological changes occurring in the early stage of T cell activation, e.g. influx of extracellular Ca$^{2+}$, is facilitated.

We initiated this study for investigating contributing roles of CD28/B7-1 and LFA-1/ICAM-1 interactions in a series of molecular changes in the early stage of T cell activation. We have shown before that the sole interaction of 2C TCR with L$^d$-QL9 complex expressed in L$^d$B7-1ICAM-1 pMVs is necessary and sufficient to induce a near maximum level of F-actin polymerization; in that process, CD28 and LFA-1 played little role (14). Different from in F-actin polymerization, both CD28 and LFA-1 took a significant and comparable part in phosphorylation of Akt (Fig. 1A and supplemental Fig. S1), conforming to their roles in activation of PI3K signaling cascades reported by others (23, 24). They also exerted comparable effects on phosphorylation of PLC-γ1 (Fig. 1B and supplemental Fig. S1). Considering that PLC-γ1 phosphorylation at Tyr$^{783}$ is mediated by the Syk family PTK, i.e. ZAP-70 in T cells, one may reason that CD28 and LFA-1 may take part in activation of ZAP-70 (25). Alternatively, it is also plausible that CD28/B7-1 and LFA-1/ICAM-1 interactions make contributions to the phosphorylation of PLC-γ1 via up-regulation of PI3K activity through which more phosphatidylinositol 3,4,5-trisphosphate becomes available. As a result, functionality of signaling molecules containing the PH domain is escalated facilitating formation of signalosome centered by PLC-γ1 and phosphorylation of PLC-γ1 by ZAP-70 (26, 27).

In striking contrast to roles in the PLC-γ1 phosphorylation, roles of CD28 and LFA-1 in the increase of cytosolic [Ca$^{2+}$] were clearly distinct (Fig. 2). Considering that phosphorylation of PLC-γ1 at Tyr$^{783}$ is reportedly critical for activation of its enzymatic activity (19), those results were intriguing and suggested a novel Ca$^{2+}$ entry mechanism exerted by LFA-1 other than SOCE. Supporting that notion, the LFA-1-dependent Ca$^{2+}$ entry proceeded without mobilization of measurable levels of intracellular Ca$^{2+}$ (Fig. 3A). The lack of intracellular Ca$^{2+}$ mobilization during culture of 2C T cells with L$^d$B7-1ICAM-1 pMVs was ensured by the result that pre-culture of 2C T cells with the QL9-loaded pMVs in the calcium-free medium did not alter either onset or magnitude of intracellular Ca$^{2+}$ mobilization by thapsigargin (supplemental Fig. S3).

Studies for SOCE have been routinely conducted following Ab cross-linking of TCRs, which elicits strong Signal 1; the TCR-mediated signaling mechanisms are collectively termed as Signal 1 (28). Reflecting the importance of Signal 1 in mobilization of intracellular Ca$^{2+}$, a transient but distinct level of intracellular Ca$^{2+}$ mobilization was readily detected when 2C T cells were cultured with pMVs expressing an elevated level of L$^d$ class I MHC (Fig. 3B). The mobilization of intracellular Ca$^{2+}$ occurred almost instantly after mixing of 2C T cells with the pMVs and peaked within a minute of the culture. In line with this, an increase in cytosolic [Ca$^{2+}$] in 2C T cells cultured with the same pMVs in Ca$^{2+}$-containing medium progressed much more promptly than with L$^d$B7-1ICAM-1 pMVs.

Experiments using wild type and LFA-1$^{-/−}$ 2C T cells cultured with L$^d$(high)B7-1ICAM-1 pMVs loaded with titrated concentrations of QL9 peptide confirmed that significance of the LFA-1/ICAM-1 interaction in extracellular Ca$^{2+}$ entry was determined by the level of cognate pMHC expression in the pMVs (Fig. 4). Under conditions that mobilization of intracellular Ca$^{2+}$ was detected, namely when 2C T cells were cultured with the pMVs loaded with high concentrations of QL9 peptide, extracellular Ca$^{2+}$ entry occurred without involvement of LFA-1/ICAM-1 interaction even though the Ca$^{2+}$ entry proceeded more progressively in the presence of LFA-1/ICAM-1 interaction. Extracellular Ca$^{2+}$ entry could still occur under a condition that intracellular Ca$^{2+}$ mobilization was not detected. In that condition, extracellular Ca$^{2+}$ entry was totally dependent on LFA-1/ICAM-1 interaction.

It is of interest that LFA-1-dependent Ca$^{2+}$ entry still relies on activity of PLC-γ1 (Fig. 5) because the level of PLC-γ1 phosphorylation at Tyr$^{783}$ showed little correlation with the level of cytosolic Ca$^{2+}$ increase (Figs. 1 and 2). PLC-γ1 plays multiple roles in T cell activation. Hydrolysis of phosphatidylinositol 4,5-bisphosphate catalyzed by PLC-γ1 results in production of diacylglycerol (DAG) as well as IP$_{3}$. DAG is imperative for activation of a family of protein kinase C and small GTP-binding proteins such as Rap1 (29). Activation of Rap1 by DAG may hold significant implications in LFA-1-dependent Ca$^{2+}$ entry because of its role in inside-out signaling for LFA-1 activation (7, 30). Thus, inhibition of LFA-1-dependent Ca$^{2+}$ entry by the PLC-γ1 inhibitor (U73122) could be explained by inhibition of DAG production by which LFA-1 activation process is impeded, and consequently signaling cascades promoted by high affinity/avidity LFA-1/ICAM-1 interaction are silenced (31).

In addition to Ca$^{2+}$ channels involved in SOCE (e.g. ORAI-1), other types of Ca$^{2+}$ channels are also expressed in T cells, e.g. L-type Ca$^{2+}$ channel and transient receptor potential channel (TRPC) (2, 3, 32). Here we hypothesize that TRPC-mediated Ca$^{2+}$ entry mechanism may be of special relevance to LFA-1-dependent Ca$^{2+}$ entry for the following reasons. First, TRPC-mediated TCR-dependent Ca$^{2+}$ entry has been revealed in human T cells (33). Second, TRPC-mediated Ca$^{2+}$ entry has been found to operate without mobilization of Ca$^{2+}$ from the
intracellular Ca\(^{2+}\) store (34). Third, a few TRPCs have been found to be activated by DAG (35). According to recent study by Mor et al. (36), interaction of LFA-1 with ICAM-1 induces strong activation of phospholipase D to produce DAG functioning in the plasma membrane. Together, it appears possible that TCR triggering leads to activation of LFA-1 via inside-out signaling, and consequently, high affinity/avidity interaction of LFA-1 with ICAM-1 results in amplification of DAG production via activation of phospholipase D. As a result, formation of TRPC is facilitated to allow entry of extracellular Ca\(^{2+}\).

It has been perceived that enhancement of Ca\(^{2+}\) signaling by LFA-1 is attained via strengthening signaling cascades induced by the TCR/pMHC interaction, namely stable and prolonged T/AFC contact established by the LFA-1/ICAM-1 interaction (11). The results of this study appear to contradict that view and instead represent the downstream Ca\(^{2+}\) allows more sustainable TCR signaling to facilitate activation of T/APC contact established by the LFA-1/ICAM-1 interaction. The downstream Ca\(^{2+}\) by the TCR/pMHC interaction, namely stable and prolonged LFA-1 is attained via strengthening signaling cascades induced by the TCR/pMHC interaction, namely stable and prolonged T/AFC contact established by the LFA-1/ICAM-1 interaction.

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