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Cross-Linking of 4-1BB Activates TCR-Signaling Pathways in CD8$^+$ T Lymphocytes[1]

Kyung-Ok Nam,[†] Hyun Kang,[†] Su-Mi Shin,[†] Kwang-Hyun Cho,[‡] Byoungsuk Kwon,[†] Byoung S. Kwon,[†] Sung-Jin Kim,* and Hyeon-Woo Lee[2]*[†]

Cross-linking of 4-1BB, a member of the TNFR family, increased tyrosine phosphorylation of TCR-signaling molecules such as CD3ϵ, CD3ζ, Lck, the linker for activation of T cells, and SH2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76). In addition, incubation of activated CD8$^+$ T cells with p815 cells expressing 4-1BBL led to redistribution of the lipid raft domains and Lck, protein kinase C-θ, SLP-76, and phospholipase C-γ1 (PLC-γ1) on the T cell membranes to the areas of contact with the p815 cells and recruitment of 4-1BB, TNFR-associated factor 2, and phospho-tyrosine proteins to the raft domains. 4-1BB ligation also caused translocation of TNFR-associated factor 2, protein kinase C-θ, PLC-γ1, and SLP-76 to detergent-insoluble compartments in the CD8$^+$ T cells, and cross-linking of 4-1BB increased intracellular Ca^{2+} levels apparently by activating PLC-γ1. The redistribution of lipid rafts and Lck, as well as translocation of PLC-γ1, and degradation of IκB-α in response to 4-1BB were inhibited by disrupting the formation of lipid rafts with methyl-β-cyclodextrin. These findings demonstrate that 4-1BB is a T cell costimulatory receptor that activates TCR-signaling pathways in CD8$^+$ T cells. ***The Journal of Immunology,*** **2005, 174: 1898–1905.**

The activation of a naive T cell requires two signals (1): ligation of the TCR with the MHC/peptide complex on the APC and cross-linking of costimulatory receptors on the T cell with the corresponding ligands on the APC. Cross-linking of CD28 on T cells with B7-1 and/or B7-2 on the APC is one such critical costimulatory event (2, 3). Several members of the TNFR family on T cells also act as costimulatory receptors enhancing T cell responses following initial activation (4–7).

4-1BB, a T cell costimulatory receptor induced by TCR activation, is a TNFR family member that is known to evoke various T cell responses through TNFR-associated factor (TRAF)[3]-mediated activation of NF-κB, p38 MAPK, stress-activated protein kinase/JNK, or ERK1/2 (8–11). 4-1BB-mediated signaling plays a role in T cell proliferation in preventing activation-induced cell death, promoting rejection of cardiac and skin allografts, eradicating established tumors, enhancing integrin-mediated cell adhesion, and increasing T cell-cytolytic potential (12–16). We have shown recently that ligation of 4-1BB provides proliferative signals to CD3-activated CD8$^+$ and CD4$^+$ T cells that prevent their clonal deletion and enhance their expansion after TCR activation (17–19). In CD8$^+$ T cells, 4-1BB promotes survival by stimulating antiapoptotic gene expression via the IκB-α/NF-κB pathway, whereas 4-1BB-mediated expansion results from increasing cell cycle-related gene transcription and translation through the combined action of the ERK1/2 and IL-2R/PI3K/Akt/mTOR/p70^{S6K} pathways (17, 18). We performed the present study to identify those early molecular changes induced by 4-1BB in CD8$^+$ T cells that set in train the signaling pathways promoting survival and cell cycle progression described above.

We showed that ligation of 4-1BB on CD8$^+$ T cells enhanced tyrosine phosphorylation of TCR-signaling proteins and caused redistribution of lipid raft domains to the contact area between the T cells and p815 cells expressing 4-1BBL. Moreover, cross-linking of 4-1BB recruited 4-1BB, TRAF2, and TCR signal molecules into lipid rafts and increased intracellular Ca^{2+} levels. These data indicate that 4-1BB provides TCR signals to CD8$^+$ T cells.

Materials and Methods

Mice, reagents, and Abs

Male BALB/c mice were obtained from Harlan Sprague Dawley and maintained under specific pathogen-free conditions. Anti-CD3 mAb (145-2C11 clone), biotin and PE-labeled anti-CD8 mAb, Cy5-, anti-FcγR mAb (2.4G2 clone), and FITC-conjugated secondary Abs, as well as isotype control Ab, were purchased from BD Pharmingen. Agonistic anti-4-1BB mAbs (3H3 and 3E1) were kind gifts from Dr. R. Mittler of Emory University (Atlanta, GA). Anti-phospholipase C-γ1 (PLC-γ1) Abs were provided generously by Dr. Y. S. Bae (Ewha Womans University, Seoul, Korea). Mock-transfected p815 cells (p815-mock) and p815 cells transfected with mouse 4-1BBL cDNA (p815-m4-1BBL) were kindly provided by Dr. H. Yagita (Juntendo University School of Medicine, Tokyo, Japan). Streptavidin-conjugated microbeads and LS columns were purchased from Miltenyi Biotech, and all primary Abs for Western blotting and immunofluorescence staining, except 4G10 (Upstate Biotechnology) and anti-flotillin 1 mAb (BD Pharmingen), were obtained from Santa Cruz Biotechnology. Methyl-β-cyclodextrin (MCD), 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2), and cholera toxin B subunit (CTx)-FITC were purchased from Sigma-Aldrich.

*Department of Pharmacology, School of Dentistry, Kyung Hee University, Seoul, Korea; [†]Immunomodulation Research Center and Graduate Program in Immunology and Biomedicine, University of Ulsan, Ulsan, Korea; and [‡]College of Medicine and Korea Bio-MAX Center, Seoul National University, Seoul, Korea;

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[2] Address correspondence and reprint requests to Dr. Hyeon-Woo Lee, Department of Pharmacology, School of Dentistry, Kyung Hee University, Seoul 130-701, Korea. E-mail address: hyeonwoo@khu.ac.kr

[3] Abbreviations used in this paper: TRAF, TNFR-associated factor; MCD, methyl-β-cyclodextrin; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; CTx, cholera toxin B subunit; PLC-γ1, phospholipase C-γ1; PKC-θ, protein kinase C-θ; pTyr, phospho-tyrosine; IP_3, inositol 1,4,5-triphosphate; CsA; cyclosporin A; SLP-76, SH2 domain-containing leukocyte phosphoprotein of 76 kDa.

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Purification of primary CD8$^+$ T cells

Cell suspensions were prepared from the spleens and lymph nodes of BALB/c mice and incubated at 37°C for 1 h in flasks to eliminate adherent cells. CD8$^+$ T cells were then purified using the MACS magnetic separation system according to the manufacturer's instructions (Miltenyi Biotech). In brief, cells were resuspended at 10^8 cells/ml in PBS containing 5% FBS, incubated with anti-CD8 mAb conjugated with biotin, and collected by incubating with streptavidin microbeads at 4°C for 15 min. LS columns (Miltenyi Biotech) were used for selecting the CD8$^+$ T cells, which were routinely >95% pure by flow cytometry.

T cell stimulation

The purified CD8$^+$ T cells were incubated with anti-CD3 mAb (0.5 μg/ml) at 10^6 cells/well in 96-well, round-bottom microplates for 16 h. After incubation, they were stained with anti-4-1BB-FITC (3E1-FITC), and >70% were found routinely by flow cytometric analysis to express 4-1BB. After verifying that the purified CD8$^+$ T cells expressed 4-1BB, 5 μg/ml agonistic anti-4-1BB mAb (3H3) or rat IgG2a (as an isotype control) were added for the indicated times.

T cell proliferation

Purified CD8$^+$ T cells were plated at 2×10^5 cells/well in 96-well, flat-bottom microplates and stimulated by coculture with irradiated (80 Gy) p815 transfectants (2×10^4) in the presence or absence of anti-CD3 mAb. For the final 12 h of culture, they received 1 μCi/well [^{3}H]thymidine (NEN). Cellular DNA was harvested and counted by liquid scintillation spectroscopy.

Separation of Brij58-insoluble and -soluble proteins

CD8$^+$ T cells were rinsed with ice-cold PBS and lysed in ice-cold TNE buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1 mM EDTA with protease and phosphatase inhibitors) containing 0.5% Brij 58 followed by incubation on ice for 30 min. Insoluble fractions were pelleted by centrifugation at 14,000 rpm for 20 min. The supernatant was saved as a soluble fraction. The insoluble pellet was resuspended in the lysis buffer containing 60 mM *N*-octyl-β-D-glucopyranoside and 0.3% deoxycholic acid, incubated for 1 h on ice, and centrifuged at 14,000 rpm for 20 min. The supernatant was stored as an insoluble fraction.

Fractionation of low-density lipid rafts by sucrose gradients

CD8$^+$ T cells (4×10^7) were lysed in 1 ml of MNE buffer (25 mM MES (pH 6.5), 150 mM NaCl, 5 mM EDTA, 30 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 10 μg/ml each of protease inhibitors) with 1% Triton X-100 for 20 min on ice and dounced 15 times. Samples were centrifuged at $1000 \times g$ for 10 min at 4°C. The supernatants were then mixed with 80% sucrose (1 ml) and transferred to Beckman ultracentrifuge tubes. Two milliliters of 30% sucrose, followed by 1 ml of 5% sucrose in MNE buffer, were overlaid. Samples were ultracentrifuged in a Beckman SW50Ti rotor ($200,000 \times g$ for 18 h). Eleven fractions were collected from the top of the gradient to the bottom. Proteins from each fraction were precipitated with TCA and then separated by 10% SDS-PAGE. Fractions 2–5 were referred to as Triton-insoluble glycolipid-enriched membrane (rafts).

Immunopreciptation and Western blotting

The purified CD8$^+$ T cells were stimulated as described earlier, and proteins were extracted with lysis buffer (10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 5 mM EDTA, 30 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM PMSF, and protease inhibitor mixture). Equal amounts of protein were diluted with 4× SDS sample buffer, applied to SDS-PAGE gels, separated, and transferred to nitrocellulose membranes (Millipore). Proteins of interest were detected with primary Abs and secondary Ab-HRP, and bound Ab was detected by ECL (Amersham Biosciences).

Immunofluorescence staining

CD8$^+$ T cells (2×10^5 cells) activated by incubation with 0.5 μg/ml anti-CD3 mAb for 16 h were stained with CTx-FITC (5 μg/ml) for 25 min in a CO_2 incubator. The CTx-labeled cells were washed three times with PBS and incubated with 5×10^5 p815-mock or p815-m4-1BBL transfectants on poly-L-lysine-coated glass slides for 25 min in a CO_2 incubator. The CTx-labeled T cells and p815 transfectants had been pretreated with 0.5 or 1.0 μg of 2.4G2, respectively, to block Fc-mediated binding of

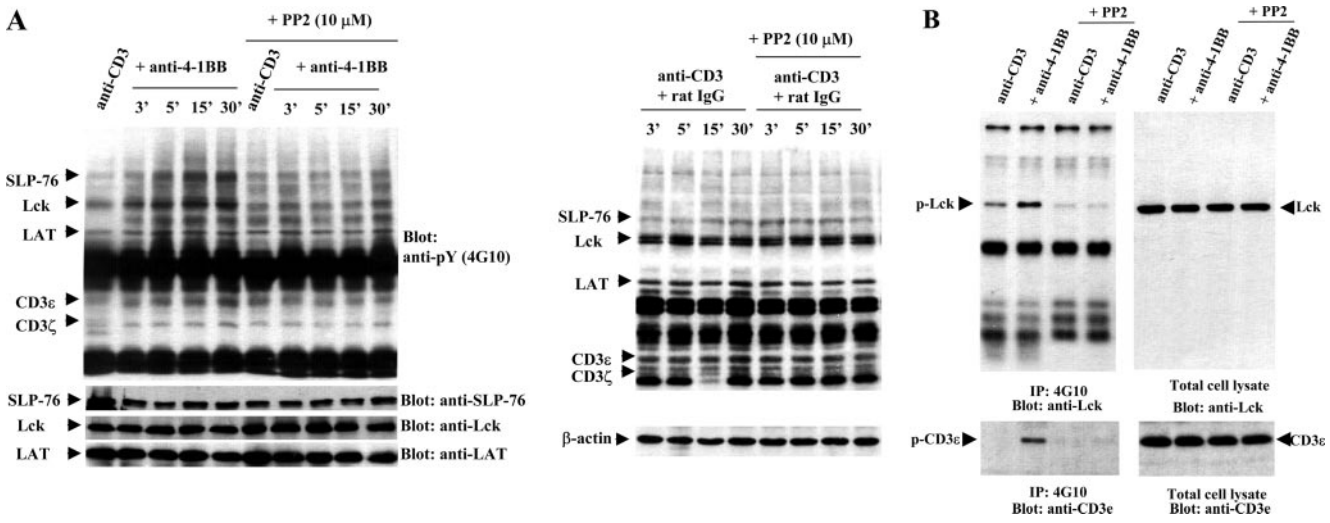

FIGURE 1. CD8$^+$ T lymphocytes (5×10^5) purified from lymph nodes and spleens of BALB/c mice with the MACS magnetic separation system were plated in a round-bottom 96-well microplate. *A*, Effect of anti-4-1BB mAb on tyrosine phosphorylation of TCR-signaling proteins. Cells were incubated with 0.5 μg/ml anti-CD3 mAb for 16 h, pretreated with 10 μM PP2 or vehicle, and then incubated with 5 μg/ml anti-4-1BB mAb or isotype control IgG2a (15 min) for the indicated times. Equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose membranes, and pTyr proteins were detected with anti-pTyr mAb (4G10). After stripping the membranes, SLP-76, linker for activation of T cells (LAT), Lck, CD3ϵ, and CD3ζ were detected by reprobing with the corresponding Abs. Similar results were obtained in three independent experiments (*left*). Cells were incubated with 0.5 μg/ml anti-CD3 mAb for 16 h, pretreated with 10 μM PP2 or vehicle, and then incubated with isotype control IgG2a for the indicated times. Equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose membranes, and pTyr proteins were detected with 4G10. After stripping the membranes, β-actin was detected by reprobing with anti-β-actin Ab. Similar results were obtained in three independent experiments (*right*). *B*, Effect of anti-4-1BB mAb on tyrosine phosphorylation of Lck and CD3ϵ. Cells were incubated with 0.5 μg/ml anti-CD3 mAb for 16 h, pretreated with 10 μM PP2 or vehicle for 30 min, and then incubated with 5 μg/ml anti-4-1BB mAb or isotype control for 15 min. Equal amounts of protein were immunoprecipitated with 4G10, immunoprecipitated proteins separated by SDS-PAGE, and transferred to nitrocellulose membranes. Lck (*upper left panel*) and CD3ϵ (*lower left panel*) were detected with anti-Lck or anti-CD3ϵ Abs. As loading controls, total proteins from cell lysates were separated on SDS-PAGE and transferred to nitrocellulose membranes. Lck (*upper right panel*) and CD3ϵ (*lower right panel*) were detected with anti-Lck or anti-CD3ϵ Abs.

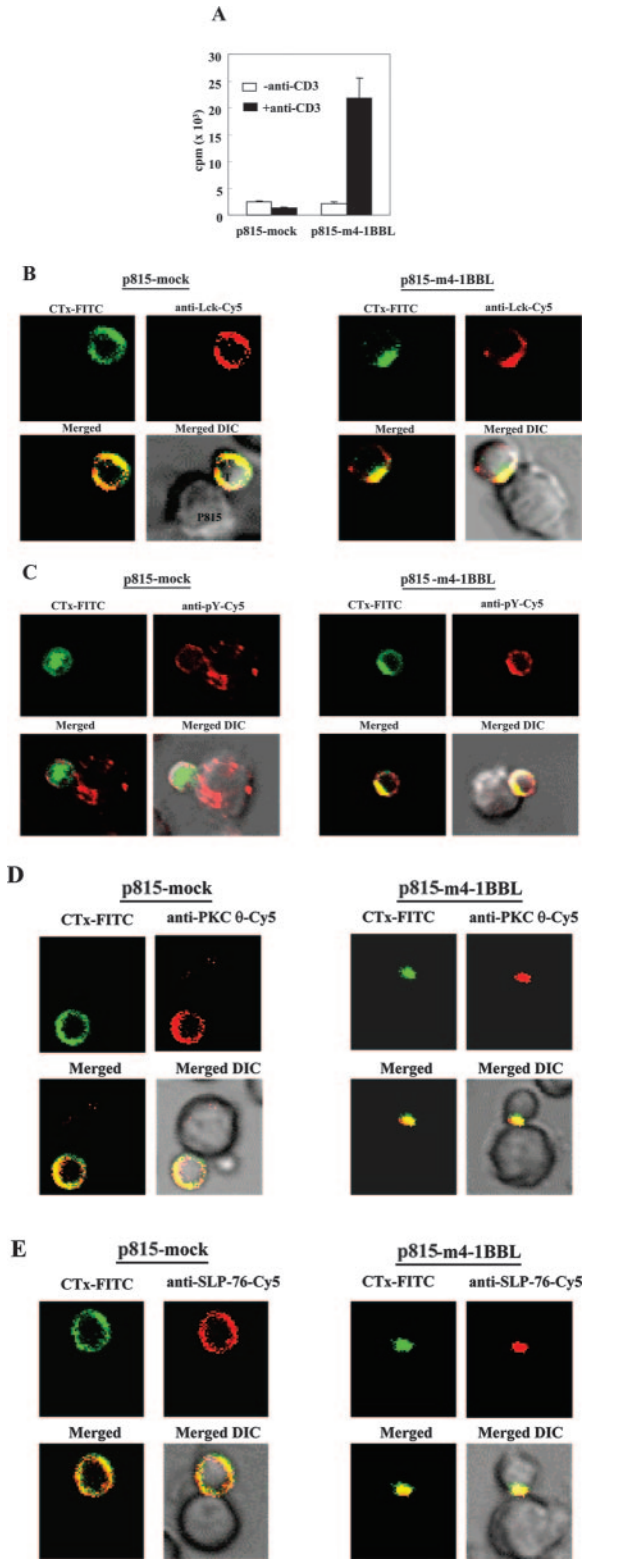

FIGURE 2. Incubation of CD8$^+$ T cells with p815-m4-1BBL transfectants redistributes lipid rafts, Lck, pTyr, PKC-θ, and SLP-76 proteins. *A*, Purified CD8$^+$ T cells exposed to (■) or not exposed to (□) anti-CD3 were incubated with irradiated p815-mock or p815-m4-1BBL cells for 3 days. During the last 12 h of culture, they received 1.0 μCi/well [^{3}H]thymidine and were harvested and counted by liquid scintillation spectroscopy. The results are means ± SD of triplicate samples. Similar results were obtained in at least six independent experiments. *B* and *C*, Purified CD8$^+$ T cells were incubated with anti-CD3 for 16 h and labeled with CTx-FITC as described in *Materials and Methods*. CTx-labeled CD8$^+$ T cells were incubated with p815-mock (*left panel*) or p815-m4-1BBL (*right panel*) cells for 25 min, stained with anti-Lck Ab (*B*) or anti-pTyr Ab (*C*), and dual-visualized by confocal microscopy. Total 150 CD8$^+$ T cells were analyzed, and 96 cells were coupled with p815 transfectants. Fifty-five percent of cell couples showed relocation of CTx-bound raft and Lck to the contact area following the treatment of p815–4-1BBL transfectants, whereas 4% of cell couples did it following the treatment of p815-mock transfectants. For p-Tyr staining, 87 cells were coupled, and 49% of cell couples showed relocation of CTx-bound raft and pTyr to the contact area following the treatment of p815-4-1BBL transfectants, whereas 3% of cell couples did it following the treatment of p815-mock transfectants. Similar results were obtained in four independent experiments. *D* and *E*, Purified CD8$^+$ T cells were incubated with anti-CD3 for 16 h and labeled with CTx-FITC as described in *Materials and Methods*. CTx-labeled CD8$^+$ T cells were incubated with p815-mock (*left panel*) or p815-m4-1BBL (*right panel*) cells for 25 min, stained with anti-PKC-θ Ab (*D*) or anti-SLP-76 Ab (*E*) and dual-visualized by confocal microscopy. Total 180 CD8$^+$ T cells were analyzed, and 76 cells were coupled with p815 transfectants. Sixty-eight percent of cell couples showed relocation of CTx-bound raft and PKC-θ to the contact area following the treatment of p815-4-1BBL transfectants, whereas 6% of cell couples did it following the treatment of p815-mock transfectants. For SLP-76 staining, 85 cells were coupled, and 56% of cell couples showed relocation of CTx-bound raft and SLP-76 to the contact area following the treatment of p815-4-1BBL transfectants, whereas 7% of cell couples did it following the treatment of p815-mock transfectants. Similar results were obtained in three independent experiments.

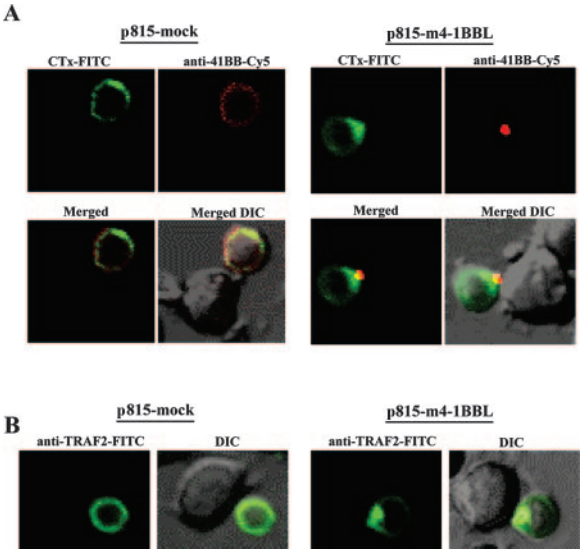

FIGURE 3. 4-1BB ligation by p815-m4-1BBL redistributes lipid rafts, 4-1BB and TRAF2, on the T cell membrane. *A*, Purified CD8$^+$ T cells were incubated with anti-CD3 Ab for 16 h and labeled with CTx-FITC as described in *Materials and Methods*. CTx-labeled CD8$^+$ T cells were incubated with p815-mock (*left panel*) or p815-m4-1BBL (*right panel*) cells for 25 min, stained with anti-4-1BB Ab (3E1), and dual-visualized by confocal microscopy. A total of 94 CD8$^+$ T cells was analyzed, and 56 cells were coupled with p815 transfectants. Sixty-two percent of cell couples showed relocation of CTx-bound raft and 4-1BB to the contact area following the treatment of p815-4-1BBL transfectants, whereas 3% of cell couples did it following the treatment of p815-mock transfectants. A representative of three independent experiments is shown. *B*, Purified CD8$^+$ T cells were treated with anti-CD3 Ab for 16 h and incubated with p815-mock (*left panel*) or p815-m4-1BBL (*right panel*) cells for 25 min, stained with anti-TRAF2 Ab, and visualized by confocal microscopy. A total of 88 CD8$^+$ T cells was analyzed, and 43 cells were coupled with p815 transfectants. Sixty-nine percent of cell couples showed relocation of TRAF2 to the contact area following the treatment of p815-4-1BBL transfectants, whereas 2% of cell couples did it following the treatment of p815-mock transfectants. A representative of three independent experiments is shown.

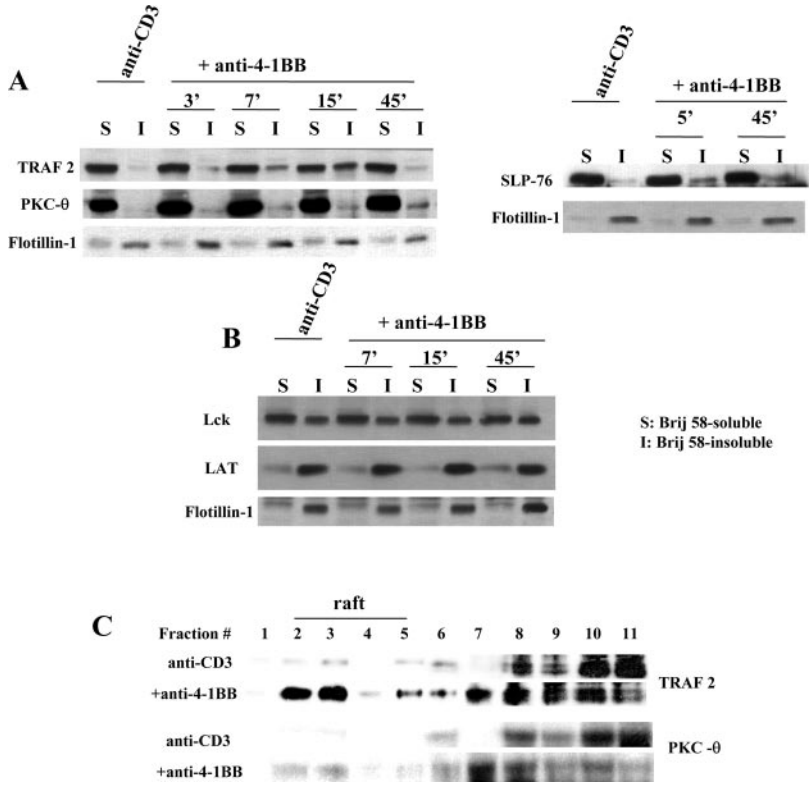

FIGURE 4. Cross-linking of 4-1BB translocates TRAF2, PKC-θ, and SLP-76 from the detergent-soluble to -insoluble fractions. *A*, Purified CD8$^+$ T cells were treated with anti-CD3 mAb for 16 h and then incubated with isotype control (15 min) or anti-4-1BB mAb for the indicated times. Brij 58-soluble and -insoluble proteins were extracted as described in *Materials and Methods*, separated by SDS-PAGE, and transferred to nitrocellulose membranes. In the *left panel*, TRAF2 was detected with anti-TRAF2 Ab. After stripping the membrane, PKC-θ and flotillin-1 were detected by reprobing with anti-PKC-θ Ab and anti-flotillin Ab, respectively. In the *right panel*, SLP-76 was detected with anti-SLP-76 Ab. After stripping the membrane, flotillin-1 was detected by reprobing with anti-flotillin Ab. Similar results were obtained in three independent experiments. *B*, Purified CD8$^+$ T cells were treated with anti-CD3 mAb for 16 h and then incubated with isotype control (15 min) or anti-4-1BB mAb for the indicated times. Brij 58-soluble and -insoluble proteins were extracted as described in *Materials and Methods*, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Lck was detected with anti-Lck Ab. After stripping the membrane, linker for activation of T cells (LAT) and flotillin-1 were detected by reprobing with anti-LAT Ab and anti-flotillin Ab, respectively. Similar results were obtained in three independent experiments. *C*, Purified CD8$^+$ T cells were treated with anti-CD3 mAb for 16 h and then incubated with isotype control or anti-4-1BB mAb for 15 min. Triton X-100-soluble and -insoluble fractions were isolated as described in *Materials and Methods*, separated by SDS-PAGE, and transferred to nitrocellulose membranes. TRAF 2 and PKC-θ were detected with anti-TRAF 2 Ab and anti-PKC-θ Ab. Similar results were obtained in three independent experiments.

anti-CD3 mAb to FcγR-bearing cells such as p815 cells before mixing them on glass slides. The cells were washed with PBS, fixed in 4% paraformaldehyde for 5 min at room temperature, permeabilized with 0.05% Triton X-100 for 30 min at room temperature, and stained with a 1/100 dilution of primary Ab for 30 min. After three washes with PBS, the cells were stained with the appropriate secondary Ab for 30 min and washed three times with PBS. A coverslip was applied with glycerol vinyl alcohol mounting solution (Zymed Laboratories), and the cells were visualized with an Olympus FV500 confocal laser scanning microscope (Olympus). To minimize cross-talk of the dual colored samples between channels, we used a sequential scanning technique exciting one dye at a time.

Imaging of cytosolic Ca^{2+}

Ca^{2+}-imaging experiments were performed with anti-CD3-treated CD8$^+$ T cells loaded with the Ca^{2+} indicator fluo3-AM (Molecular Probes). Cultures were incubated for 25 min at 37°C in the presence of 5 μM fluo3-AM, followed by two washes with washing buffer (PBS containing 0.1% BSA). The cells were attached to glass coverslips, which were mounted under a purpose-built chamber such that washing buffer containing stimulating compounds could be added. In most experiments, intracellular calcium was analyzed immediately with an Olympus FV500 confocal laser scanning microscope. Images were acquired at 25°C at one frame per 1.1 s for 31.2 min in the presence of each stimulus.

Results

Cross-linking of 4-1BB activates TCR-mediated signals in primary CD8$^+$ T lymphocytes

To study 4-1BB-mediated early signaling pathways in mouse CD8$^+$ T cells expressing 4-1BB, we first purified CD8$^+$ T cells using the MACS separation system, as described in *Materials and Methods*. The CD8$^+$ T cells were then incubated with a suboptimal concentration (0.5 μg/ml) of anti-CD3 mAb for 16 h and expression of 4-1BB verified by flow cytometry (17, 18). To examine the effect of cross-linking of 4-1BB on phospho-tyrosine (pTyr) proteins, the activated CD8$^+$ T cells were incubated with 5 μg/ml agonistic anti-4-1BB mAb (3H3) or isotype control rat IgG2a in the presence or absence of 10 μM PP2 (a specific Src protein kinase inhibitor) for the indicated times. Cross-linking of 4-1BB with 3H3 increased the phosphorylation on tyrosine residues in several proteins, whereas the treatment with isotype control did not affect it (Fig. 1*A*). By stripping and reprobing the same membrane with appropriate Abs, we showed that these proteins were TCR-signaling components such as SH2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76), Lck, linker for activation of T cells, CD3ϵ, and CD3ζ (Fig. 1*A*). Pretreatment of the CD8$^+$ T

cells with PP2 completely abolished this 4-1BB-induced tyrosine phosphorylation. To confirm this, total cell lysates were immunoprecipitated with anti-pTyr Ab (4G10), and the immunoprecipitated proteins were separated by SDS-PAGE and blotted with anti-Lck Ab or anti-CD3ϵ Ab. As shown in Fig. 1*B*, 4-1BB engagement stimulated tyrosine phosphorylation of Lck and CD3ϵ. These data indicate that cross-linking of 4-1BB stimulates TCR-signaling pathways.

Engagement of 4-1BB by 4-1BB ligand leads to reorganization of lipid rafts and Lck and recruitment of pTyr proteins into the lipid rafts

To examine possible changes in the distribution of lipid rafts, Lck, pTyr proteins, protein kinase C-θ (PKC-θ), and SLP-76, we incubated naive $CD8^+$ T cells with anti-CD3 Ab for 16 h to induce 4-1BB expression and then mixed CTx-FITC-labeled $CD8^+$ T cells with p815-m4-1BBL or p815-mock. Following this, the cells were stained with anti-Lck, anti-pTyr, anti-PKC-θ, or anti-SLP-76 Abs, and the secondary Ab was conjugated with Cy5 and observed by confocal microscopy. Fig. 2*A* shows that the p815-m4-1BBL transfectants enhanced the proliferation of $CD8^+$ T cells only if the latter were preincubated with anti-CD3 Ab, indicating that the p815-m4-1BBL transfectants can cross-link 4-1BB on the $CD8^+$ T cells and induce 4-1BB-mediated signaling. Cross-linking of 4-1BB on the $CD8^+$ T cells by p815-m4-1BBL redistributed dispersed lipid membrane rafts to the area of contact between the $CD8^+$ T cells and the p815-m4-1BBL transfectants (Figs. 2, *B–D*, and 3*A*). Lck, pTyr proteins, PKC-θ, and SLP-76 were also found within the lipid rafts following 4-1BB engagement (Fig. 2, *B–D*).

4-1BB ligation recruits 4-1BB and TRAF2 into lipid rafts

We next examined changes in the distribution of 4-1BB and TRAF2 on $CD8^+$ T cells following cross-linking of 4-1BB. When $CD8^+$ T cells were incubated with p815-mock, 4-1BB was dispersed over the cell membrane. However, incubation with p815-m4-1BBL led to its recruitment to the lipid rafts in the area of contact between the $CD8^+$ T and p815-m4-1BBL cells (Fig. 3*A*). Because TRAF2 is known to mediate 4-1BB signaling by interacting with the cytoplasmic domain of 4-1BB (9), we investigated the effect of cross-linking 4-1BB on the localization of TRAF2. TRAF2 proteins were also found to reorganize to the contact area following engagement of 4-1BB with p815-m4-1BBL (Fig. 3*B*).

Cross-linking of 4-1BB triggers translocation of TRAF2 and TCR-signaling molecules to detergent-insoluble fractions

To test the redistribution of TRAF2 and TCR-signaling proteins, we separated detergent-soluble and -insoluble membrane fractions of $CD8^+$ T cells using Brij 58 and examined the location of the proteins in the detergent-soluble and -insoluble fractions. Following cross-linking of 4-1BB, TRAF2 and TCR-signaling proteins such as SLP-76 and PKC-θ were translocated from the Brij 58-soluble to the insoluble fraction (Fig. 4*A*), supporting the conclusion that cross-linking of 4-1BB redistributes membrane lipid rafts and translocates 4-1BB, TRAF2, and TCR-signaling proteins to them. These early events appear to be responsible for 4-1BB-evoked TCR signaling. Fig. 4*B* shows that Lck and the linker for activation of T cells were located in the insoluble fraction, regardless of whether 4-1BB was cross-linked. These proteins are known to be located in lipid rafts and to move to the contact area between T cells and APC together with the lipid rafts following stimulation with Ag (20). To confirm recruitment of PKC-θ and TRAF2 to lipid rafts by cross-linking of 4-1BB, we fractionated low-density rafts by sucrose gradients and ultracentrifugation, separated proteins of each fraction by SDS-PAGE, and then performed Western blot analysis. Fig. 4*C* shows that PKC-θ and TRAF-2 were translocated to low-density lipid raft fractions upon 4-1BB engagement.

4-1BB ligation increases intracellular Ca^{2+}

Because cross-linking of TCR with Ag or anti-CD3 mAb increases intracellular Ca^{2+} via TCR-signaling pathways, if 4-1BB engagement enhances or initiates TCR-signaling pathways, it should also increase intracellular Ca^{2+} levels. To examine this possibility, we labeled $CD8^+$ T cells with fluo3-AM and measured cytosolic Ca^{2+} levels by confocal microscopy. As expected, incubation of naive $CD8^+$ T cells with anti-CD3 mAb evoked a rapid rise in intracellular Ca^{2+} (Fig. 5*A*). When naive $CD8^+$ T cells were preincubated with anti-CD3 mAb to induce expression of 4-1BB and exposed to anti-4-1BB mAb, cross-linking of 4-1BB with anti-4-1BB mAb rapidly increased intracellular Ca^{2+} (Fig. 5*B*). Cross-linking of the TCR recruits PLC-γ1 to lipid rafts and activates it, and this in turn produces inositol 1,4,5-triphosphate (IP_3), which activates the IP_3 receptors on the endoplasmic reticulum and stimulates Ca^{2+} release (21). Fig. 6*A* shows that cross-linking of 4-1BB also recruits PLC-γ1 to the Brij 58-insoluble fraction of the cell membrane. This suggests that the 4-1BB-mediated increase in intracellular Ca^{2+} is also caused by activation of PLC-γ1 and the subsequent action of IP_3 on its receptors on the endoplasmic reticulum.

MCD blocks 4-1BB-mediated signaling

Depletion or sequestration of cholesterol with MCD inhibited translocation of PLC-γ1 to the detergent-insoluble membrane fraction (Fig. 6*A*) and CTx-stained lipid rafts (Fig. 6*B*) in response to

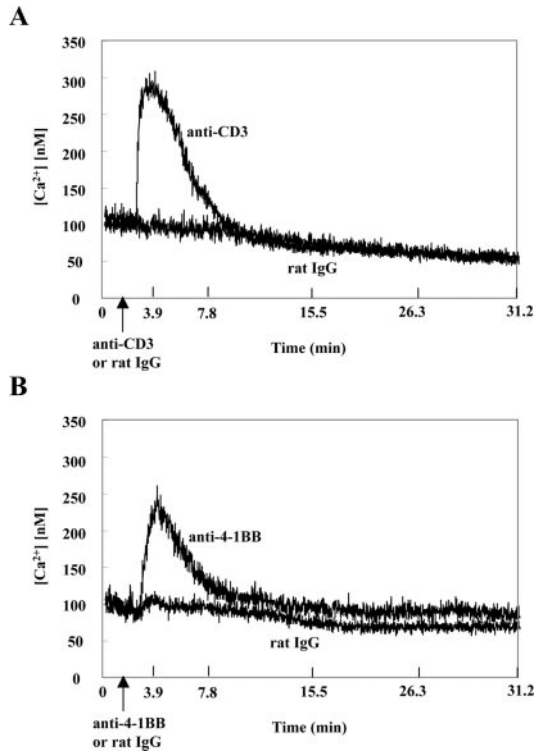

FIGURE 5. Cross-linking of 4-1BB mobilizes intracellular Ca^{2+}. *A*, Purified $CD8^+$ T cells were loaded with fluo3-AM, washed, and monitored for intracellular Ca^{2+} by confocal microscopy for 31.2 min in the presence of anti-CD3 or isotype control. *B*, Purified $CD8^+$ T cells were treated with anti-CD3 mAb for 16 h and loaded with fluo3-AM. The fluo3-AM-loaded $CD8^+$ T cells were washed, and their intracellular Ca^{2+} was monitored by confocal microscopy for 31.2 min in the presence of anti-4-1BB mAb or isotype control. Results are representative of three independent experiments.

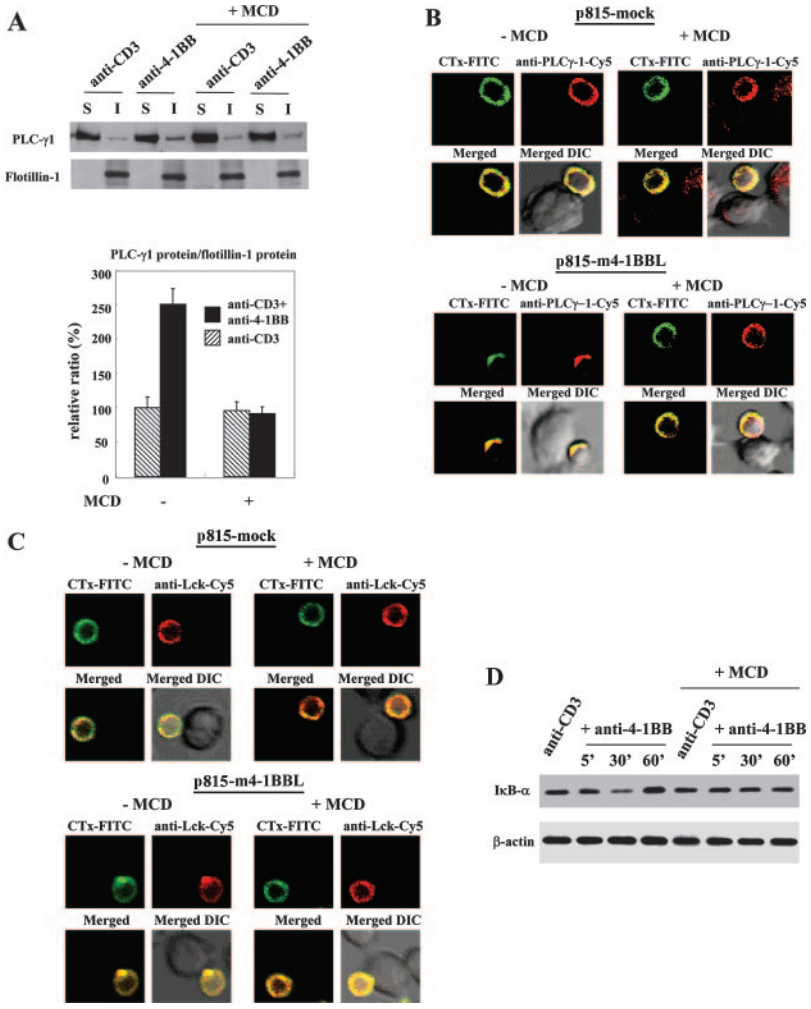

FIGURE 6. *A*, 4-1BB ligation leads to translocation of PLC-γ1 to Brji-58-insoluble fractions. Purified $CD8^+$ T cells were treated with anti-CD3 mAb for 16 h and then incubated with isotype control or anti-4-1BB mAb for 5 min in the presence or absence of MCD. Brij 58-soluble and -insoluble proteins were extracted as described in *Materials and Methods*, separated by SDS-PAGE, and transferred to nitrocellulose membranes. PLC-γ1 was detected with anti-PLC-γ1 Ab. After stripping the membrane, flotillin-1 was detected by reprobing with anti-flotillin-1 Ab. Densitometric analyses are presented as the relative ratio of PLC-γ1 to flotillin protein. The relative ratio measured as 100 is arbitrarily presented. Bars represent the mean ± SD from three experiments. *B*, MCD inhibits redistribution of lipid rafts and PLC-γ1 following engagement of 4-1BB by 4-1BBL. Purified $CD8^+$ T cells were incubated with anti-CD3 for 16 h and labeled with CTx-FITC as described in *Materials and Methods*. The labeled cells were either pretreated with 15 mM MCD (*right panel*) or kept untreated (*left panel*) and then incubated for 5 min with p815-mock (*top panel*) or p815-m4-1BBL cells (*bottom panel*). The cells were then stained with anti-PLC-γ1 Ab and dual-visualized by confocal microscopy. A total of 108 $CD8^+$ T cells was analyzed, and 68 cells were coupled with p815 transfectants. Fifty-eight percent of cell couples showed relocation of CTx-bound raft and PLC-γ1 to the contact area following the treatment of p815-4-1BBL transfectants, whereas <6% of cell couples did it following the treatment of p815-mock or p815-4-1BBL transfectants in the presence of MCD. Similar results were obtained in four independent experiments. *C*, MCD inhibits redistribution of lipid rafts and Lck following engagement of 4-1BB by 4-1BBL. Purified $CD8^+$ T cells were incubated with anti-CD3 for 16 h and labeled with CTx-FITC as described in *Materials and Methods*. The labeled cells were either pretreated with 15 mM MCD (*right panel*) or kept untreated (*left panel*) and then incubated for 25 min with p815-mock (*top panel*) or p815-m4-1BBL cells (*bottom panel*). The cells were then stained with anti-Lck Ab and dual-visualized by confocal microscopy. A total of 93 $CD8^+$ T cells was analyzed, and 59 cells were coupled with p815 transfectants. Fifty-three percent of cell couples showed relocation of CTx-bound raft and Lck to the contact area following the treatment of p815-4-1BBL transfectants, whereas <3% of cell couples did it following the treatment of p815-mock or p815-4-1BBL transfectants in the presence of MCD. Similar results were obtained in four independent experiments. *D*, MCD blocks 4-1BB-induced IκB-α degradation. Purified $CD8^+$ T cells were treated with anti-CD3 mAb for 16 h and incubated with isotype control (5 min) or anti-4-1BB mAb for the indicated times in the presence or absence of 15 mM MCD. Total cell lysates were extracted as described in *Materials and Methods*, and proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. IκB-α was detected with anti-IκB-α Ab. After stripping the membrane, β-actin was detected by reprobing with anti-β-actin Ab. Similar results were obtained in three independent experiments.

4-1BB. It also blocked redistribution of lipid rafts and of Lck following cross-linking of 4-1BB by 4-1BBL (Fig. 6*C*). We have reported that cross-linking of 4-1BB leads to degradation of IκB-α and activation of NF-κB, which in turn up-regulates the antiapoptotic genes, *bcl-*x_L and *bfl-1*, and enhances survival of the $CD8^+$ T cells (17). Fig. 6*D* shows that 4-1BB-induced IκB-α degradation is blocked by MCD, suggesting that activation of late signaling pathways, including those involving NF-κB and ERK1/2, by 4-1BB is due to the reorganization of lipid rafts and the activation of TCR-signaling pathways.

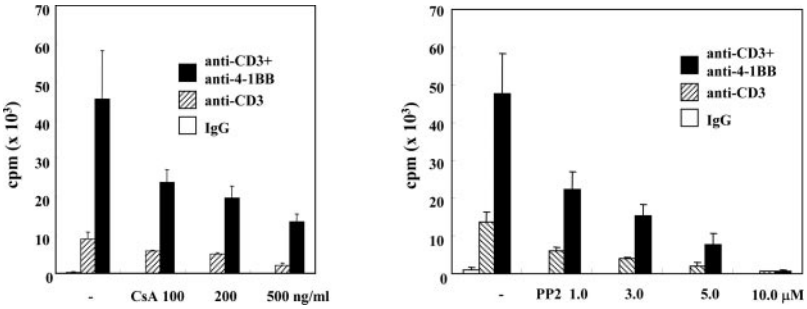

FIGURE 7. 4-1BB-induced proliferation is blocked by CsA and PP2. After incubation with anti-CD3 mAb for 16 h, the cells were pretreated with vehicle, CsA (100–500 ng/ml), or PP2 (1–10 μM) for 1 h and then with 5 μg/ml anti-4-1BB mAb or isotype control for 48 h. During the last 12 h of culture, the cells were pulsed with 1.0 μCi/well [^{3}H]thymidine. All cells were harvested and counted by liquid scintillation spectroscopy. The results are represented as means ± SD of triplicates. Similar results were obtained in three independent experiments.

Cyclosporin A (CsA) or PP2 blocks 4-1BB-mediated proliferation of CD8$^+$ T lymphocytes

To test whether CsA, a Ca^{2+}/calmodulin-dependent calcineurin inhibitor, or PP2 blocks 4-1BB-mediated proliferation, CD8$^+$ T cells were treated with anti-CD3 mAb plus rat IgG2a or anti-CD3 mAb plus anti-4-1BB mAb in the presence or absence of indicated concentration of CsA or PP2. As shown in Fig. 7, CsA or PP2 suppressed the proliferation enhanced by 4-1BB engagement, although they also inhibited the proliferation of CD8$^+$ T cells by CD3 engagement. It is because Lck, a Src protein kinase, and calcineurin, a Ca^{2+}/calmodulin-dependent phosphatase, are pivotal proteins for TCR-mediated signaling pathways.

Discussion

4-1BB transmits a potent costimulatory signal to both CD8$^+$ and CD4$^+$ T cells, promoting their expansion, survival, differentiation, and cytokine expression (19, 22–29). We have shown that the cellular responses mediated by 4-1BB depend on late 4-1BB-evoked signaling proteins such as NF-κB and ERK1/2 (17, 18). In the present work, we have presented several findings related to the early 4-1BB-signaling pathways in CD8$^+$ T cells. First, we showed that cross-linking of 4-1BB in primary CD8$^+$ T lymphocytes increased tyrosine phosphorylation of TCR pathway proteins. Because pretreatment of CD8$^+$ cells with PP2, which blocks Src tyrosine kinases such as Lck and Fyn (30), completely abolished this 4-1BB-evoked tyrosine phosphorylation, it was clearly the result of tyrosine phosphorylation of one of the early TCR pathway proteins by Lck. Second, we demonstrated that cross-linking of 4-1BB on CD8$^+$ T cells in p815-m4-1BBL transfectants caused the redistribution of lipid rafts to the area of contact of the T cells with the p815-m4-1BBL cells. TCR pathway proteins such as Lck, pTyr, PKC-θ, and SLP-76 were also redistributed to the lipid rafts, and other TCR pathway proteins such as PKC-θ and SLP-76 were translocated from the Brij 58-soluble to the insoluble fraction, indicating that these proteins were also recruited to the lipid rafts. Third, cross-linking of 4-1BB recruited 4-1BB itself to lipid rafts in the area of cell contact. TRAF2 was also recruited, as shown by confocal microscopy, and by its appearance in the Brij 58-insoluble fraction. Fourth, cross-linking of 4-1BB increased intracellular Ca^{2+}, apparently due to translocation of PLC-γ1 to the lipid rafts and its subsequent activation. Fifth, MCD, which disrupts raft formation (31), blocked translocation of PLC-γ1 to the detergent-insoluble fraction, redistribution of lipid rafts and of Lck, and degradation of IκB-α as a result of cross-linking of 4-1BB. Finally, PP2, a Src kinase inhibitor, or CsA, a calcineurin inhibitor, suppressed 4-1BB-mediated proliferation. CsA completely blocks 4-1BB-induced IL-2 and IFN-γ mRNA expression (unpublished data). Because we have observed similar 4-1BB effects in CD4$^+$ T cells (data not shown), these could be early molecular events underlying the role of 4-1BB in survival and cell cycle progression in both CD8$^+$ and CD4$^+$ T cells (17–19).

This is the first report that shows that 4-1BB, a TNFR family member, activates TCR-signaling pathways. 4-1BB is known to evoke a variety of cellular responses via TNFR-signaling pathways (8–11). It activates NF-κB, p38 MAPK, stress-activated protein kinase/JNK, and ERK1/2 via TRAF/TNFR-associated death domain protein in T cells, and it is generally accepted that its cross-linking recruits or stimulates the TNFR signalosome by which 4-1BB activates downstream signaling. The mechanism(s) by which cross-linking of 4-1BB activates TCR signaling remains to be uncovered. There are several plausible mechanisms. First, because 4-1BB increases the adhesion of T cells to extracellular matrix proteins (32, 33), its ligation could extend the duration of interaction between TCR and Ag/MHC as a result of increased adhesion of the T cells to the APC. Second, recruitment of TRAF2 to the raft fraction could initiate tyrosine phosphorylation and activation of Lck via an interaction of TRAF2 with an unidentified protein kinase. Third, the binding of 4-1BB to TRAF2 upon 4-1BB engagement (9) could redistribute lipid rafts, which might by default relocate Lck to the cell contact area. Lck, in turn, could be activated by the same route as that responsible for stimulation of the TCR by Ags or by anti-CD3 Ab. It has been reported that cross-linking CD40, another member of TNFR family, recruits CD40, TRAF2, and TRAF3 in addition to recruiting Lyn Src family kinase to lipid rafts and initiating tyrosine phosphorylation of intracellular protein substrates. This promotes ERK1/2 and p38 MAPK activation, which leads to the production of various cytokines in dendritic cells (34). Recruitment of CD40, TRAFs, and Lyn to lipid rafts following CD40 ligation may be a prerequisite for the effects of tyrosine phosphorylation of intracellular proteins on subsequent signaling pathways and cytokine production. Finally, 4-1BB-mediated activation of classical signal transduction pathways could modulate TCR-signaling proteins and initiate or enhance TCR-signaling cascades.

It has been suggested that 4-1BB-mediated CD8$^+$ T cell survival is due to up-regulation of bcl-x_L and bfl-1 via the NF-κB pathway in response to cross-linking of 4-1BB (13). In the hands of others, cell cycle progression of CD8$^+$ T cells in response to treatment with agonistic 4-1BB mAb was enhanced by both 4-1BB-mediated ERK1/2 activation and 4-1BB-evoked IL-2-mediated signaling pathways (14). We believe that our demonstration that cross-linking of 4-1BB recruits 4-1BB and TRAF2 to lipid rafts and activates TCR-signaling pathways accounts for how engagement of 4-1BB activates NF-κB and ERK1/2 and enhances the survival and expansion of CD8$^+$ T lymphocytes. This mechanism may account for the clonal expansion and enhanced survival of CD8$^+$ T lymphocytes following Ag challenge in vivo.

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