CD99 activates T cells *via* a costimulatory function that promotes raft association of TCR complex and tyrosine phosphorylation of TCR ζ

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Abstract

We investigated the co-stimulatory role of a cell-surface protein, CD99. Co-ligation of CD99 and suboptimal CD3 induced T-cell activation to a level comparable to that obtained with optimal CD3 or CD3+CD28. We also noted concomitant enhancement of the earliest T-cell receptor (TCR) signaling events. In addition, co-ligation of CD99 and CD3 led to translocation of TCR complexes into the lipid raft, without concomitant migration of CD99 to the raft, and consequent enhancement of TCR ζ-mediated signal 1. These data demonstrate the unique properties of CD99 co-stimulation that distinguish this molecule from CD28 and other raft-resident co-stimulatory factors.

Keywords: antigens; lymphocyte activation; receptors, antigen, T-cell; signal transduction; T lymphocytes

Introduction

The physiological process of T-cell activation can be divided into a series of temporal stages consisting of initial adhesion, initial signaling (formation of the contact cap), and sustained signaling (Bromley *et al.*, 2001). During these stages, two independent signals are required for the enactment of full T-cell activation: (1) specific interaction of a T-cell receptor (TCR) with a cognate peptide/MHC on APCs, and (2) the ligation of surface co-stimulatory molecules with specific ligands on APCs. Among the known co-stimulatory molecules, CD28 launches the most effective activation of T cells (Alegre *et al.*, 2001; Frauwirth and Thompson, 2002). However, recent reports have suggested that many other candidates, most of which reside in the lipid raft, induce T-cell activation as potently as CD28 does (Denning *et al.*, 1988; Ledbetter *et al.*, 1988; Green *et al.*, 1994; Tai *et al.*, 1996; Yashiro-Ohtani *et al.*, 2000; Stillwell and Bierer, 2001).

The lipid raft is a unique membrane microdomain that is enriched with specific types of lipids and cholesterol and is associated with many key molecules involved in TCR signaling. Upon engagement of cognate ligands (peptide/MHC), TCRs are recruited toward the raft along with a series of tyrosine-phosphorylated signaling molecules (Moran and Miceli, 1998; Xavier *et al.*, 1998; Janes *et al.*, 1999). The lipid raft thus serves as a stage for protein-protein interactions among many surface molecules, thereby instigating a series of signaling cascades following TCR engagement (Cherukuri *et al.*, 2001).

CD99, a monomeric 32-kDa transmembrane glycoprotein, is the product of *mic2* and lacks significant homology to any functional or structural domains of known proteins (Schenkel *et al.*, 2002). Ligation of CD99 with anti-CD99 mAb induces homotypic aggregation and apoptosis of thymocytes (Bernard *et al.*, 1997) as well as up-regulation of cell-surface proteins, such as TCR and MHC classes I and II (Choi *et al.*, 1998). A recent report suggested that reorganization of the cytoskeleton is involved in the function of CD99 (Cerisano *et al.*, 2004).

In this study, we explored the possible role and mechanism of CD99 as a co-stimulatory molecule in T-cell activation. CD99 engagement leads to the

recruitment of TCR/CD3 into lipid rafts. However, CD99 enhances T-cell activation through increasing signal 1, such as phosphorylation of TCR ζ, only when a suboptimal level of signal is provided by TCR/CD3 ligation. Our results indicate another role of CD99 in T cells, i.e., co-stimulation, the underlying mechanism of which differs in many ways from those of other co-stimulatory molecules involved in T-cell activation.

Materials and Methods

Cell culture

Jurkat T cells (clone E6-1, obtained from American Type Culture Collection; Rockville, MD) were maintained in culture in RPMI-1640 supplemented with 5% FCS, 2 mM L-glutamine, 0.1% penicillin/streptomycin. Human PBMC were isolated from heparinized blood of healthy adult donors by standard density gradient centrifugation with Ficoll-Paque (Amersham Pharmacia; Uppsala, Sweden). Isolated PBMC were maintained in culture in RPMI-1640 supplemented with 10% autologous serum.

Antibodies

The following mAbs were used in this study: anti-CD99 (DiNonA; Seoul, Korea), biotinylated or FITC labeled anti-CD3 (UCHT1, DiNonA), goat or rabbit anti-mouse secondary antibodies (DiNonA), anti-CD3 and CD1a (OKT3 and OKT6 respectively for cell stimulation; American Type Culture Collection), anti-CD3 ε (M-20 for immunoblotting, Santa Cruz Biotechnology; Santa Cruz, CA), anti-CD3 ζ (6B10.2, Santa Cruz Biotechnology), anti-phosphotyrosine (4G10, Upstate Biotechnology; Lake Placid, NY), anti-transferrin receptor (16E, a generous gift from Junji Kato, Fourth Department of Internal Medicine, Sapporo Medical University School of Medicine, Sapporo, Japan), anti-lck (3A5, Santa Cruz Biotechnology) and anti-CD28 (CD28.2, BD Pharmingen; San Diego, CA).

Proliferation assays

Proliferation assays were performed using standard techniques (Waclavicek *et al.*, 1998). Briefly, 10^5 PBMC/well were cultured for 72 h in mAb-coated 96-well plates in RPMI media supplemented with autologous sera, and pulsed with a [^{3}H] thymidine solution (1 μCi/well, 6.7 Ci/mmol specific activity, Amersham; Aylesbury, UK) for the last 18 h before harvesting. The mean incorporation of thymidine in DNA was measured in quadruplicate wells by liquid scintillation counting.

TCR down-regulation

PBMC or Jurkat cells were incubated at 37°C in wells previously coated with desired mAbs for the indicated time (Kim *et al.,* 2004; Zhang *et al.,* 2005). The cells then were resuspended, washed and stained with the specific Abs directly labeled with FITC or PE. The mean fluorescence intensity of each sample was measured at each point by FACScalibur flow cytometer (BD Bioscience; San Jose, CA).

Isolation of lipid rafts

Lipid rafts were isolated using modified lysis conditions and flotation on discontinuous sucrose gradients (Montixi *et al.*, 1998). Briefly, Jurkat T cells were lysed and the lysed solution was further homogenized with 10 strokes in a Wheaton loose-fitting dounce homogenizer. Nuclei and cellular debris were pelleted and the supernatant was adjusted to 1.33 M sucrose by adding 2 ml of 2 M sucrose prepared in buffer TNEV (50 mM TrisCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1 mM Na_3VO_4), placed at the bottom of an ultracentrifuge tube, and overlayed with a step sucrose gradient (0.2-0.9 M with 0.1 M steps, 1 ml each) in buffer TNEV. The tube was centrifuged at 38,000 r.p.m. for 16 h in an SW41 rotor (Beckman Instruments Inc; Palo Alto, CA) at 3°C, and 1 ml fractions were harvested from the top.

Immunoprecipitation, and Western blot analysis

For immunoprecipitations (Jang *et al.*, 2004), total cell lysates were pre-cleared and then incubated overnight at 4°C with antibody coupled to protein A beads (Pierce; Rockford, IL). Protein samples were resolved on 12% SDS-PAGE and visualized using ECL kit (Amersham; Little Chalfont, Buckinghamshire, UK).

Immunofluorescence microscopy

Jurkat T cells were stimulated with mAb-coated beads on the slides, and rinsed in PBS and fixed in 4% paraformaldehyde for 20 min. After quenching paraformaldehyde with 50 mM NH_4Cl, the cells were permeabilized in 0.1% saponin for 10 min, and blocked with a solution of 0.01% saponin and 1% BSA. Cholera toxin-B-FITC/biotin, anti-CD3-biotin and anti-CD99-FITC were used to label the conjugates.

Results

Enhanced activation of T cells by co-ligation of CD99 and CD3

The co-stimulatory function of CD99 in T-cell acti-

vation was demonstrated by a [^{3}H]-thymidine incorporation assay using PBMC cultured in the presence of anti-CD99 mAb (DN16) plus various concentrations of anti-CD3 mAb (OKT3). Co-ligation of CD99 and CD3 markedly enhanced cell activation, allowing treated cells to proliferate and secret IL-2 at suboptimal concentrations of anti-CD3 (Figure 1A and B). Enhanced cell activation by co-ligation of CD99 was also evident from the increased surface expression of the activation marker CD69 and the
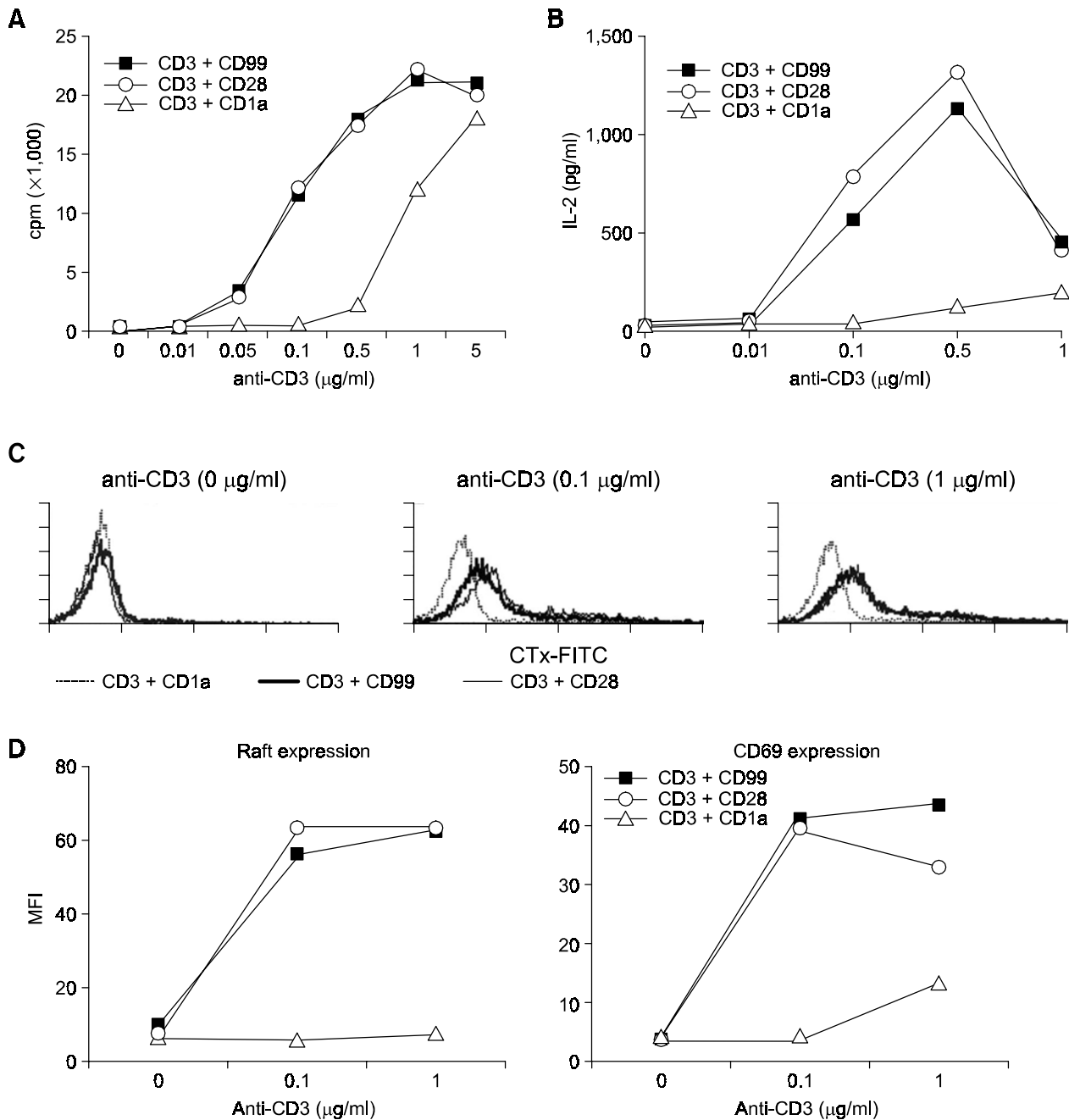
Figure 1. CD99 engagement facilitates a T-cell response in the presence of suboptimal CD3 stimulation. (A, B) Human PBMC were stimulated in 96-well plates with anti-CD3 together with anti-CD99, CD28, or CD1a (an isotype-matched control) for 72 h. (C) Human PBMC were incubated in 24-well plates coated with varying concentrations of anti-CD3 in combination with the indicated antibodies and stained with anti-CD69-FITC/CD4+CD8-PE or CTx-FITC/CD4+CD8-PE and analyzed for the surface expression of CD69 or GM1 in the live-gated T-cell fraction. (D) Quantitation of Figure 1C. Representative data from three independent experiments are shown.

lipid raft (Figure 1C and D). Together, these results indicate that CD99 acts as a co-stimulatory molecule that sensitizes cells for activation through CD3 ligation.

CD99 co-ligation amplifies signal 1

Co-stimulatory molecules in T cells can be categorized according to whether they amplify the generation of signal 1 or signal 2 (adhesion versus co-stimulation). In addition, the level of TCR down-regulation reflects the number of functionally triggered TCRs and thus the amount of signal 1 (Valitutti *et al.*, 1995). To test whether CD99 ligation plays a role in generating signal 1, the level of TCR down-regulation after co-ligation of CD3 and CD99 was examined. Interestingly, TCR stimulation with anti-CD3+CD99 resulted in a two- to four-fold greater down-regulation of TCR than obtained with anti-CD3 alone or anti-CD3 + CD28 (Figure 2A). The diffe-

rence in TCR down-regulation was most obvious at a low concentration of anti-CD3 (Figure 2B). This was also the case in a Jurkat T-cell line (Figure 2C).

TCR/CD3 and CD99 co-engagement enhances tyrosine phosphorylation of the TCR ζ-chain
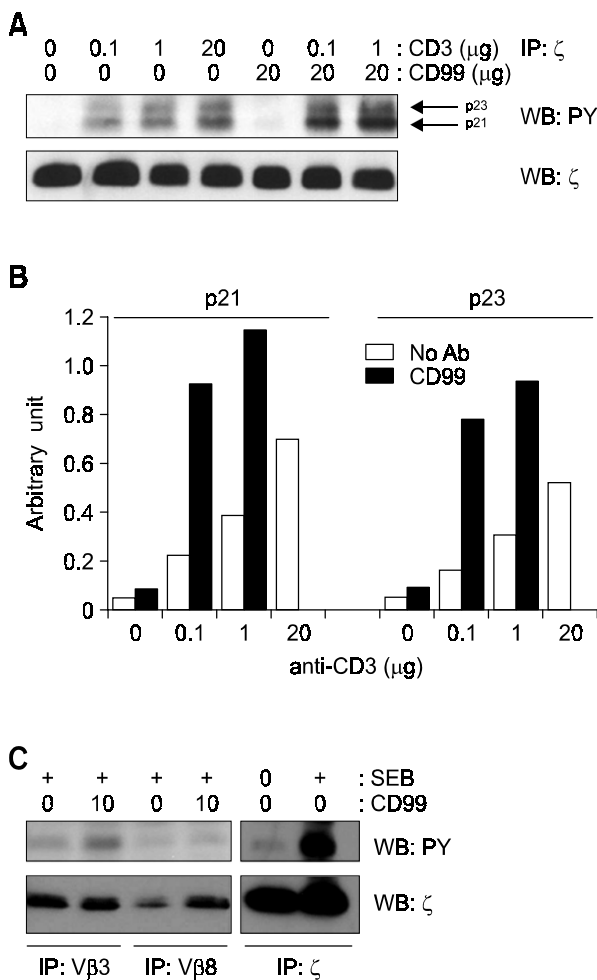
The ability of CD99 co-ligation to increase cell proliferation and accentuate signal 1 suggests that CD99 affects TCR signal strength by increasing the efficiency of the earliest signaling events generated upon TCR engagement. This hypothesis was tested by treating Jurkat T cells with anti-CD99 plus anti-CD3 and then comparing the extent of TCR ζ phosphorylation. Co-ligation of CD99 with suboptimal CD3 induced tyrosine phosphorylation of TCR ζ as did cells triggered by optimal engagement of CD3 (Figure 3A). The ratio of p-TCR ζ to total TCR ζ after CD99 co-ligation with suboptimal CD3 were approximately three- to four-fold higher than those ob-
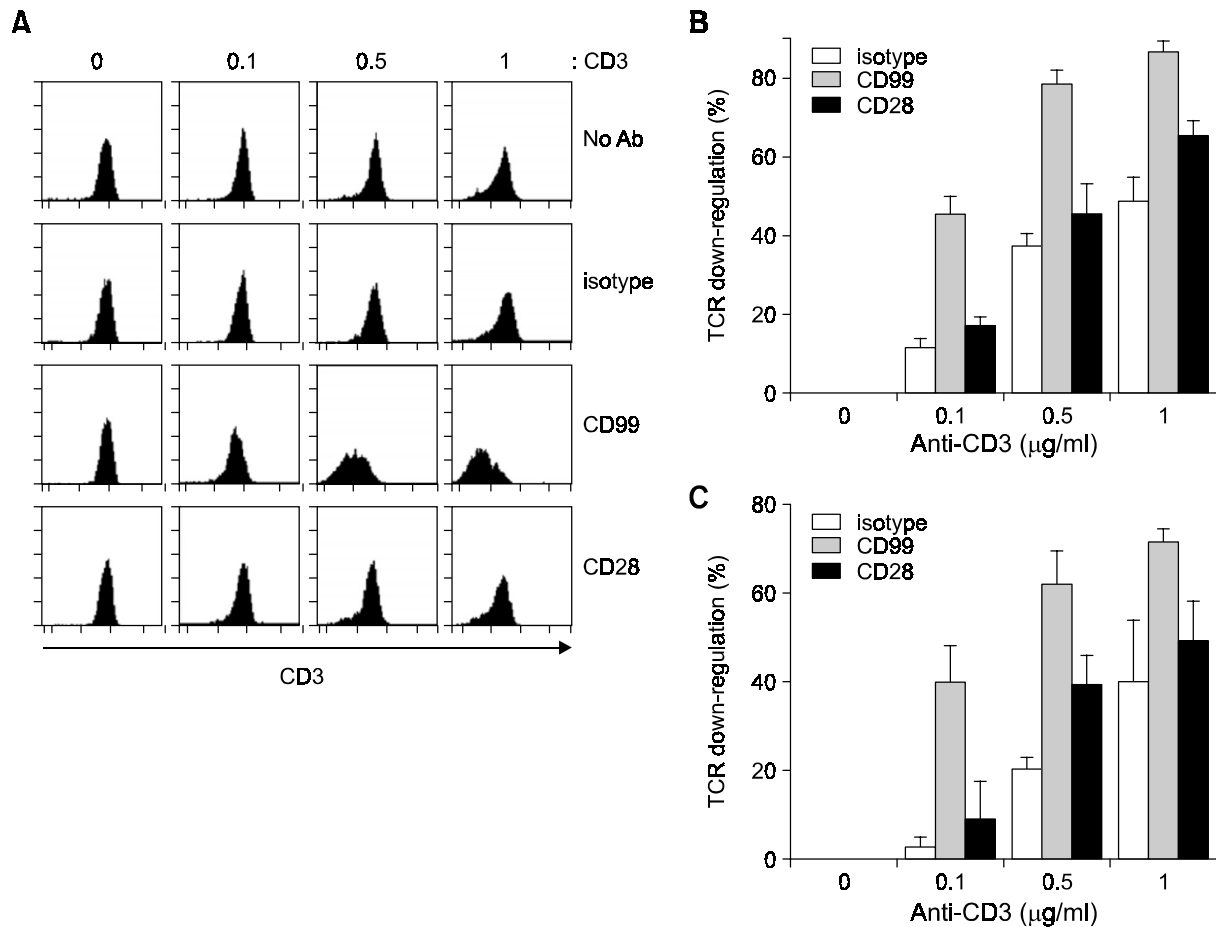
Figure 2. Enhanced TCR down-regulation after stimulation with anti-CD3 plus CD99. Human PBMC (A, B) or Jurkat cells (C) were treated as in Figure 1, and TCR expression was assessed 4 h later by flow cytometry. TCR down-regulation (%) was calculated as a normalized value as follows: 100 × (MFI of TCR in resting cells - MFI of TCR in stimulated cells)/MFI of TCR in resting cells (B and C). MFI, Mean fluorescence intensity.

served after cell treatment with suboptimal doses of anti-CD3 only (Figure 3B).

To exclude any possible artificial effect of antibody ligation, the same study was repeated with TCR-Vβ3-transfected Jurkat cells after stimulation with the superantigen SEB. Vβ3-expressing Jurkat cells express two different TCR sets: transfected Vβ3 and the endogenously expressed Vβ8 family. When Vβ3-expressing Jurkat cells were stimulated with soluble

SEB in the presence of CD99 engagement, the level of ζ-chain phosphorylation of Vβ3 was greater than in cells stimulated with soluble SEB only. In contrast to Vβ3-ζ, the tyrosine residues on the Vβ8-ζ were not phosphorylated (Figure 3C). These data show that CD99-induced co-stimulation is specific for TCR engaged with SEB or anti-CD3 mAb.

CD99 is weakly associated with the raft on resting and activated T cells

Several studies have reported that some proteins residing in the raft fraction, such as CD5 and CD9, exhibit a co-stimulatory capacity by enhancing TCR signaling. To test whether CD99 belongs to these raft-resident co-stimulators, cell lysates of Jurkat T
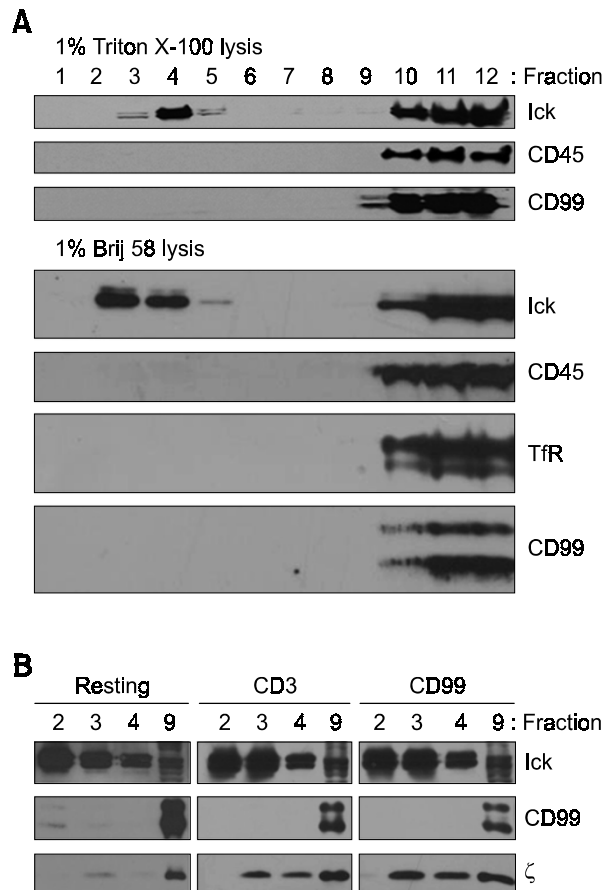

Figure 3. Enhanced phosphorylation of TCR ζ in cells co-stimulated by TCR and CD99 engagement. Jurkat T cells were stimulated for 5 min with varying concentrations of anti-CD3 mAb with or without anti-CD99 mAb. (A) Cells were subsequently lysed and immunoprecipitated with anti-ζ mAb. The precipitates were immunoblotted with anti-phosphotyrosine (4G10) mAb (top panel), stripped, and re-probed with anti-ζ mAb as a loading control (bottom panel). (B) Densitometry of the phosphorylated (p23, p21) vs. non-phosphorylated ζ-chains. (C) Jurkat cells expressing both Vβ3 and Vβ8 were activated with soluble SEB plus anti-CD99 and treated as above except that immunoprecipitations were done with anti-Vβ mAbs. Representative data from three independent experiments are shown. PY, Phosphotyrosine; IP, immunoprecipitation; WB, Western blotting.

Figure 4. Distribution pattern of CD99 in resting and activated T cells. (A) Jurkat cells were treated with 1% Triton X-100 (top) or Brij 58 lysis buffer (bottom) for 30 min on ice and then subjected to sucrose density gradient ultracentrifugation. Fractions were collected and equivalent portions of each fraction were analyzed by immunoblotting with the corresponding mAbs. (B) T cells were stimulated with irrelevant control mAb, anti-CD3 or anti-CD99, and lysed with 1% Brij 58 lysis buffer. Protein bands were analyzed by probing with corresponding Abs. Representative data from three independent experiments are shown.

cells were fractionated and the presence of CD99 in the raft fractions was checked by immunoblot analysis. We could detect CD99 marginally in lipid rafts of either resting or activated T cells, which suggests none of stimulation conditions was able to coax the transport of CD99 molecules into raft fractions at a readily detectable level (Figure 4). The fragile association of CD99 molecules in the lipid raft clearly distinguishes CD99 from other raft-resident co-stimulatory molecules.

CD99 engagement leads to translocation of the TCR complex into lipid rafts and facilitates TCR ζ phosphorylation in the presence of suboptimal CD3 stimulation

The fact that CD99 engagement generates active phosphorylation of TCR ζ as well as aggregation of

lipid rafts on the cell surface suggests that the CD99 signal augments the signal generated by suboptimal levels of CD3 ligation via transporting TCR into the raft area. Therefore, we examined the presence of TCR ζ and ε chains in the raft fractions of cells stimulated with various combinations of anti-CD3 and CD99. Interestingly, the translocation of TCR ζ and ε chains into the rafts was observed when CD99 ligation was included in the stimulation roster (Figures 4B and 5A). The amounts of TCR ζ and ε chains found in the raft fractions were almost similar to those in cells stimulated by optimal CD3 ligation or suboptimal CD3 plus CD99 co-ligation (Figure 5A and B). In contrast, in the case of CD28, neither CD28 single-ligation nor CD3+CD28 co-ligation induced translocation of TCR into lipid rafts.

To confirm the above data in intact cells, the positions of rafts and CD3 molecules in the sti-
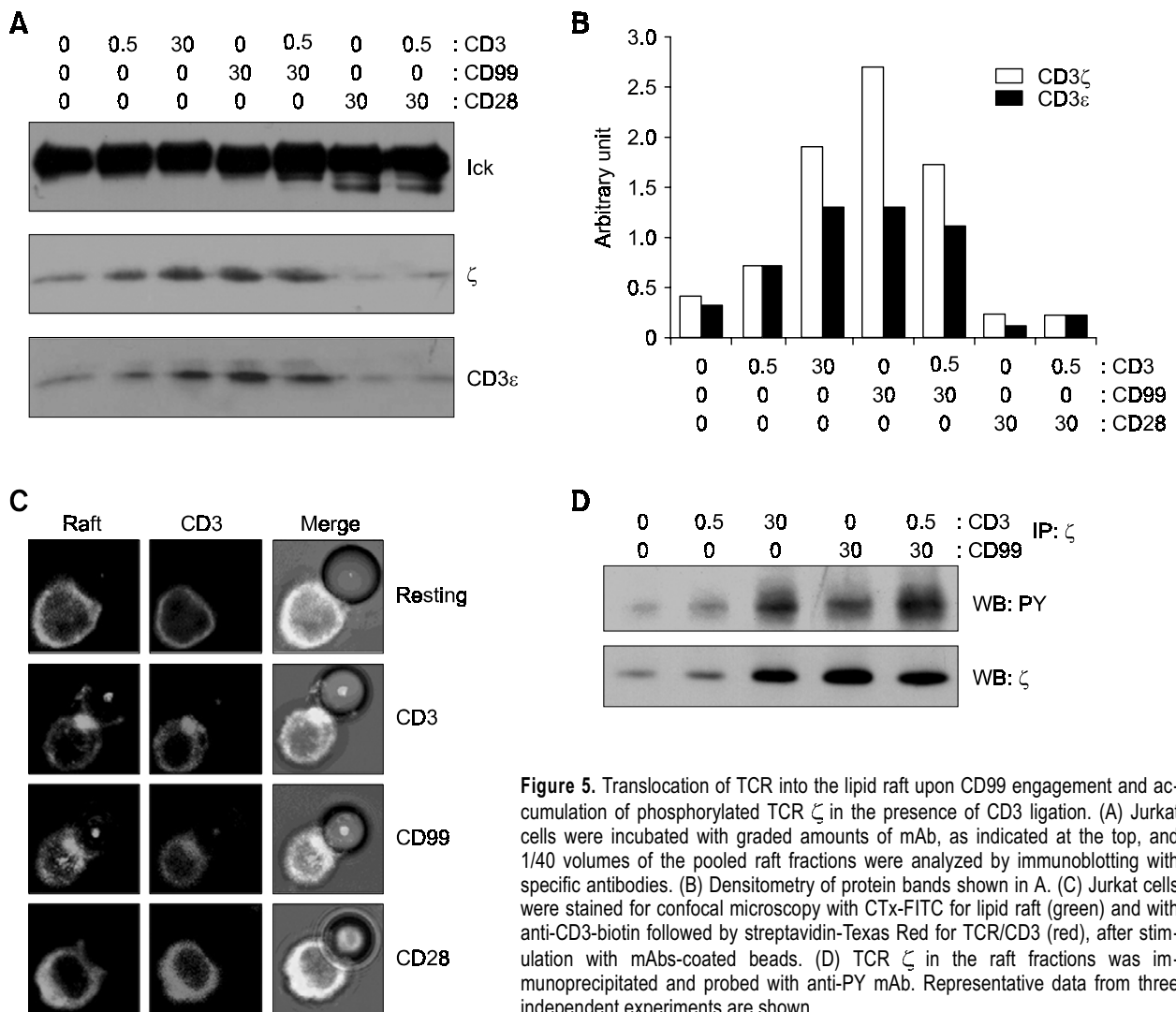
Figure 5. Translocation of TCR into the lipid raft upon CD99 engagement and accumulation of phosphorylated TCR ζ in the presence of CD3 ligation. (A) Jurkat cells were incubated with graded amounts of mAb, as indicated at the top, and 1/40 volumes of the pooled raft fractions were analyzed by immunoblotting with specific antibodies. (B) Densitometry of protein bands shown in A. (C) Jurkat cells were stained for confocal microscopy with CTx-FITC for lipid raft (green) and with anti-CD3-biotin followed by streptavidin-Texas Red for TCR/CD3 (red), after stimulation with mAbs-coated beads. (D) TCR ζ in the raft fractions was immunoprecipitated and probed with anti-PY mAb. Representative data from three independent experiments are shown.

mulated cells were visualized by confocal micro-scopy. Consistent with the biochemical data, the engagement of cells with beads coated with anti-CD99 (100 mg/ml) induced the redistribution and co-localization of CD3 molecules and rafts to the cell-to-bead contact sites, demonstrating that CD99 simulation indeed prompts the translocation of TCR ζ into lipid rafts (Figure 5C).

To confirm whether the lipid raft indeed serves as the transduction site of the CD99 co-stimulatory function, the level of tyrosine phosphorylation on raft-bound TCR ζ was compared after stimulating cells with CD99 ligation alone or with co-ligation of CD99 and CD3. The presence of phosphorylated ζ chain was readily detected in cells given a subop-timal dose of anti-CD3 in conjunction with anti-CD99 (Figure 5D). Although a significant number of TCR ζ molecules were present in the rafts in the case of CD99 single-ligation, the extent of TCR ζ phos-phorylation (assessed by the relative intensity of p-TCR ζ vs. that of total TCR ζ) under this condition was not much higher than in resting cells or in cells stimulated only with a suboptimal amount of anti- CD3. These results demonstrate that CD99 is capable of amplifying weak signals generated by suboptimal CD3 ligation to a level corresponding to that induced by optimal CD3 ligation, by increasing the local concentration of signaling molecules in the raft.

Discussion

We have demonstrated that the cell-surface protein CD99 acts as a co-stimulatory molecule in T-cell activation. The co-stimulatory function of CD99 was executed by enhancing the association of TCR ζ with lipid rafts. Upon additional stimulation with CD3, these raft-associated TCR complexes were exten-sively phosphorylated.

The degree of T-cell activation achieved by CD99 co-stimulation was comparable to that of CD28 (Figure 1). Previous reports have also shown the induction of IL-2 promoter activity by CD99 and CD28 (Waclavicek *et al.*, 1998; Zhou *et al.*, 2002). However, the inability of CD28 to affect TCR signal 1 and to translocate TCR ζ chains into rafts (Figures 2 and 5) clearly distinguishes the controlling mechanism of CD99 in the immune response from that of CD28. Furthermore, co-stimulation of CD28 differentiates naïve T cells into the Th2 subtype (Shahinian *et al.*, 1993; Skapenko *et al.*, 2001), whereas CD99 co-stimulation induces the produc-tion of Th1-type cytokines in polyclonally activated T-cell lines (Waclavicek *et al.*, 1998). These data suggest that CD99 provides a signal different from that of CD28, supporting previous notions that CD28

signaling is not related directly to TCR-derived sig-nals (Reinhold *et al.*, 1997; Alegre *et al.*, 2001; Michel *et al.*, 2001; Frauwirth and Thompson, 2002).

Other non-CD28 co-stimulatory molecules such as CD2 (Bierer *et al.*, 1988), CD5 (Ledbetter *et al.*, 1986), CD9 (Tai *et al.*, 1996), and CD44 (Huet *et al.*, 1989), also have a co-stimulatory effect on resting T cells as potently as that of CD28 in the presence of suboptimal doses of anti-CD3 mAb. Co-ligation of these molecules and CD3 leads to increased Ca^{2+} mobilization, association of TCR/CD3 with rafts, and increased phosphorylation of LAT, compared to single ligation of CD3 (Yashiro-Ohtani *et al.*, 2000). Yet, a major difference between these molecules and CD99 is their residency in the rafts of resting T cells or of cells engaged with CD2 ligand (Yang and Reinherz, 2001; Badour *et al.*, 2003). The fact that most CD99 molecules are far removed from the raft, and that even those few residing inside the raft maintain only a weak and fragile association under any circumstance (Figure 4), draws attention to how CD99 is able to translocate TCR ζ and ε chains into rafts and increase TCR signaling. Equally intriguing is the identity of signal pathways involved in trans-ducing CD99 stimulation. We checked lck activity to test a hypothesis that CD3 plus CD99 stimulation enhances raft-associated kinase activity directly. However, we did not observe the differences bet-ween CD3 plus CD99 and CD3 alone (data not shown).

Evidence for a close relationship between CD99 and the cytoskeleton has accumulated, based on the function of CD99 as an adhesion molecule that induces homotypic cell aggregation (Bernard *et al.*, 1995). CD99 engagement increases intracellular transport of TCR and MHC molecules to the cell surface in thymocytes (Choi *et al.*, 1998) and elicits the exocytic transport of GM1 in Jurkat cells, accom-panied by the rearrangement of actin cytoskeletons (Yoon *et al.*, 2003). Furthermore, CD99 has been linked to the migration of monocytes (Schenkel *et al.*, 2002) and to morphological changes in T and B cells (Kim *et al.*, 1998; Bernard *et al.*, 2000). Thus, it is highly probable that CD99-induced co-stimulation is mediated by cytoskeletal remodeling rather than enhancing TCR signaling directly.

In conclusion, CD99 was shown to function as a co-stimulatory molecule that is able to compensate for suboptimal CD3 ligation in promoting full acti-vation of T cells; thereby amplifying signal 1. It will be highly relevant to identify potential adaptor molecules acting downstream of CD99, not only to elucidate the exact mechanism of CD99 co-stimula-tion, but also to define functional and mechanistic differences between CD99 and other co-stimulatory molecules.

Acknowledgement

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