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Orally Tolerized T Cells Can Form Conjugates with APCs but Are Defective in Immunological Synapse Formation 1

Wataru Ise,* Kentaro Nakamura,* Nobuko Shimizu,* Hirofumi Goto,* Kenichiro Fujimoto,* Shuichi Kaminogawa,*† and Satoshi Hachimura2*

Oral tolerance is systemic immune hyporesponsiveness induced by the oral administration of soluble Ags. Hyporesponsiveness of Ag-specific CD4 T cells is responsible for this phenomenon. However, the molecular mechanisms underlying the hyporesponsive state of these T cells are not fully understood. In the present study, we investigated the ability of orally tolerized T cells to form conjugates with Ag-bearing APCs and to translocate TCR, protein kinase C-θ (PKC-θ), and lipid rafts into the interface between T cells and APCs. Orally tolerized T cells were prepared from the spleens of OVA-fed DO11.10 mice. Interestingly, the orally tolerized T cells did not show any impairment in the formation of conjugates with APCs. The conjugates were formed in a LFA-1-dependent manner. Upon antigenic stimulation, the tolerized T cells could indeed activate Rap1, which is critical for LFA-1 activation and thus cell adhesion. However, orally tolerized T cells showed defects in the translocation of TCR, PKC-θ, and lipid rafts into the interface between T cells and APCs. Translocation of TCR and PKC-θ to lipid raft fractions upon antigenic stimulation was also impaired in the tolerized T cells. Ag-induced activation of Vav, Rac1, and cdc42, which are essential for immunological synapse and raft aggregation, were down-regulated in orally tolerized T cells. These results demonstrate that orally tolerized T cells can respond to specific Ags in terms of conjugate formation but not with appropriate immunological synapse formation. This may account for the hyporesponsive state of orally tolerized T cells. The Journal of Immunology, 2005, 175: 829–838.

Oral tolerance is the state of systemic Ag-specific hyporesponsiveness induced by the oral administration of soluble Ags. The physiological role of oral tolerance is thought to be the prevention of hypersensitivity to food Ags and is a representative form of peripheral tolerance against non-self-Ags under physiological conditions. In addition, a number of experimental autoimmune diseases can be inhibited by oral administration of the corresponding autoantigen (1). Therefore, it is believed that this approach could be used therapeutically to treat autoimmune, inflammatory, and allergic disorders.

Oral tolerance is mediated by T cells. Three mechanisms have been proposed to be involved in the induction of oral tolerance: 1) unresponsiveness of T cells to specific Ags (anergy) (2–4); 2) immune suppression by regulatory T cells that produce TGF-β or IL-10 (5, 6); and 3) the elimination of Ag-specific T cells via apoptosis (clonal deletion) (7). In addition to CD4 T cells, several studies have suggested the role of CD8 T cells (8, 9) or γδ T cells (10, 11) in oral tolerance, especially in terms of their immune suppressive activity. However, CD4 T cells, rather than other T cells, seem to be indispensable to oral tolerance because in vivo depletion of CD4 T cells abrogates oral tolerance induction (12, 13), and CD4 T cells can transfer oral tolerance in vivo (14).

Previous studies demonstrated defects in TCR-mediated signaling in in vivo-tolerized T cells. Upon TCR stimulation, these T cells show incomplete protein tyrosine phosphorylation of signaling molecules (15–17) and impaired nuclear translocation of transcription factors (18–20). We have recently characterized TCR-mediated signaling in orally tolerized T cells. We have used OVA-specific TCR transgenic mice and induced tolerance to peripheral CD4 T cells by the feeding of high doses of OVA. In this experimental system, orally tolerized CD4 T cells show impaired calcium/NFAT signaling upon TCR cross-linking but normal activation of the MAPK pathway (21). Furthermore, we have found that orally tolerized T cells up-regulate caspase activation and show decreased levels of caspase-targeted proteins, such as Grb2-related adaptor downstream of She (GADS) and Src homology 2 domain containing leukocyte protein of 76 kDa (SLP-76), which are important adaptor molecules in TCR signaling (22). Thus, it has been suggested that these characteristics of TCR signaling could be responsible for the hyporesponsiveness of orally tolerized T cells.

Most of these studies used artificial stimulation, such as Ab-mediated cross-linking of TCR, to elicit strong signaling and to facilitate biochemical analysis. The events occurring in tolerized T cells upon stimulation with Ag-APCs, which are more physiological ligands for TCR, have not been documented. Upon recognition of specific Ags via TCRs, T cells form conjugates with APCs. The interaction culminates in the formation of a highly organized complex of receptors, adhesion molecules, and intracellular signaling molecules at the interface between the T cells and APCs, the so-called immunological synapse (23). It is well established that upon antigenic stimulation, TCR, protein kinase C-θ (PKC-θ)3, and LFA-1 polarize to the APC interface and segregate into distinct supramolecular clusters following a precise relative topology (24, 25). This process is mediated, in part, by remodeling of the actin

1 Abbreviations used in this paper: PKC-θ, protein kinase C-θ; CTx, cholera toxin B; RBD, rap binding domain; PBD, p21-binding domain; PLC-γ, phospholipase-γ.

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cytoskeleton (26). In addition, lipid rafts containing various sig-
naling molecules are also recruited to the interface and participate in
immunological synapse formation (27, 28). Forming a long-
vived conjugate and synapse is thought to be required for full T cell
activation because the disruption of conjugate and synapse forma-
tion, by drug or Ab, results in the inhibition of proliferation and
IL-2 production (29). Specific characteristics of immunological
syndrome formation have been investigated in various cell types,
such as Th1, Th2 cells (30), CD8 T cells (31, 32), and immature
thyocytes (33, 34). However, it remains unclear whether in vivo-
tolerized T cells can form conjugates with APCs and form immu-
nological synapses at the interface.

In this article, we prepared orally tolerized CD4 T cells from
OVA-specific DO11.10 mice fed OVA and examined their ability
to form conjugates with APCs, to form immunological synapse,
and to activate signaling pathways associated with these two events.
We demonstrate that orally tolerized T cells can form sta-
ble conjugates with Ag-bearing APCs but cannot translocate TCR,
PKC-θ, and lipid rafts into the contact site. Biochemical analysis
revealed that orally tolerized T cells can activate Rap1, which is
required for integrin-mediated adhesion. However, they also
showed defects in Ag-induced activation of Vav, Rac, and cdc42,
which are critical for immunological synapse formation.

Materials and Methods

Mice

Female BALB/c mice were purchased from CLEA Japan and used at 8–10
wk old. DO11.10 TCR transgenic mice (35) were maintained by back-
crossing to BALB/c mice. Female DO11.10 mice were used at 8–12 wk
old. All mice used in this study were maintained in the animal facility at the
University of Tokyo and used in accordance with the guidelines of the
University of Tokyo.

Peptide, Abs, and reagents

OVA323–339 (ISOQVHAAHAINEAGR) was purchased from Bio-
logica. Rabjty poloconal anti-Vav, cdc42, Rap1, PKC-θ Ab, and mouse
anti-TCR-ζ mAb (B6D12.2) were purchased from Santa Cruz Biotech-
ology. Mouse anti-Rac1 mAb (10b2), unconjugated or biotin-conjugated rat
anti-LFA-1 mAb (M174), Cy3-conjugated rat anti-CD4 mAb
(H129.19), PE-conjugated rat anti-CD44 (pgp1), and FITC-conjugated
streptavadin were purchased from BD Pharmingen. PE-conjugated rat anti-
CD62L (MEL14) was purchased from ebioscience. Mouse anti-
phosphothreonine mAb (4G10) was obtained from Upstate Biotechnology.
Clontotype-specific mAb KJ1.26 was purified from ascites and conjugated
with biotin or FITC in our laboratory. PE-conjugated KJ1.26 was pur-
chased from Caltag Laboratories. Rat anti-MHC class II mAb (M5) was
purified and conjugated with FITC in our laboratory. Alexa Fluor-594-
conjugated goat anti-rabbit IgG and Alexa Fluor 647-conjugated strepta-
vidin were purchased from Molecular Probes. FITC-conjugated cholera
toxin B (CTXx) was obtained from Sigma-Aldrich. HRP-conjugated goat
anti-rabbit IgG was from Cell Signaling Technology. HRP-conjugate goat
anti-mouse IgG was purchased from Amersham Biosciences.

Immunezation and oral tolerance induction

For the induction of oral tolerance, OVA (WAKO) was administered to
DO11.10 mice in their drinking water (100 mg/ml) for 7 days. The daily
intake of OVA was estimated to be ~100 mg/mouse. For immunization,
mixtures of OVA and CFA was injected i.p. to DO11.10 mice at a
OVA dose of 100 μg/mouse. The immunized mice were sacrificed 7 days
later.

Cell preparation

Splenic CD4 T cells from DO11.10 mice were purified by positive selec-
tion using MACS CD4 microbeads (Miltenyi Biotech). The purity of iso-
lated cells was routinely >95%. T cell-depleted splenocytes, such as APCs,
were prepared from the spleens of BALB/c mice as described previously
(36). APCs used in this study were <5% Thy1.2+.

Proliferation and cytokine secretion assays

Splenic CD4 T cells from DO11.10 mice (1 × 10^5/well) were cultured with
APCs (2 × 10^5/well) in the presence or absence of various concentrations
of OVA323–339 in 96-well flat-bottom plates for indicated periods, and
proliferation was assayed by measuring the incorporation of [H]thymidine
(1 μCi/well) during the final 24 h of culture. For cytokine secretion as-
says, culture supernatants were harvested at the indicated time points. IL-2,
IL-4, and IFN-γ concentrations were determined by means of a sandwich
ELISA as described previously (36).

Flow cytometric analysis of surface molecules

For analysis of LFA-1 expression, splenic CD4 T cells from DO11.10 mice
were stained with biotin-anti-LFA-1, FITC-conjugated streptavidin, PE-
KJ1.26, and Cy3-chrome-anti-CD4. For analysis of CD62L or CD44 ex-
cvates T cells were stained with FITC-KJ1.26, Cy3-chrome-anti-CD4,
and PE-CD44 or PE-CD62L. Cells were analyzed on a flow cytometer (BD
LSR, BD Biosciences) using CellQuest software (BD Biosciences).

In vivo OVA-specific Ab production

DO11.10 mice were immunized i.p. with 100 μg of OVA in the form of an
emulsion in CFA. Seven days after, the mice were boosted with 100 μg of
OVA emulsified in IFA. Seven days after the boosting, the mice were bled,
and OVA-specific IgG titer of their sera was measured by ELISA as
described previously (37).

T-APC conjugate formation assay

APCs (2 × 10^5/well) were pulsed with the indicated concentrations of
OVA323–339 for 2 h in 96-well round-bottom plates. Splenic CD4 T cells
from DO11.10 mice were mixed with APCs at a 1:1 ratio and centrifuged
briefly. After incubation for the indicated period at 37°C, cells were vig-
rously pipetted to disrupt nonspecific conjugates. Cells were then fixed
with 2% paraformaldehyde for 10 min at room temperature. Cells were
stained with FITC-anti-MHC class II, PE-KJ1.26, and Cy3-chrome-anti-
CD4 and Abs and analyzed by flow cytometry. Conjugates were determined as
the percentage of CD4+ KJ1.26+ events that were also MHC class II+.

Confocal microscopy

Splenic CD4 T cells (5 × 10^5) from DO11.10 mice were mixed with APCs
(5 × 10^5), which had been previously pulsed with 20 μM OVA323–339
for 2–4 h, and incubated at 37°C for 30 min. For lipid raft staining, CD4
T cells were stained with FITC-CTX before incubation with APCs. Cells
were then fixed with 4% paraformaldehyde in PBS for 10 min. In the case of
PKC-θ staining, cells were permeabilized by 0.1% Triton X-100 in PBS
for 2 min. Cells were blocked overnight with 0.1% BSA/PBS and
stained with biotin-KJ1.26 followed by Alexa Fluor 647-streptavadin.
For PKC-θ staining, cells were further stained with rabbit anti-PKC-θ followed
by Alexa Fluor 594-conjugated anti-rabbit IgG. Staining was performed for
1 h each. All images were taken using a Fluoview FV500 laser scanning
confocal microscope (Olympus).

Pull-down assay, immunoprecipitation, and immuno blot

APCs were pulsed with 20 μM OVA323–339 for 4 h at 37°C, after which
they were washed three times. APCs were then fixed with 0.2% parafor-
maldehyde for 10 min to avoid activation of signaling molecules. Splenic
CD4 T cells (3 × 10^5) from DO11.10 mice were mixed with peptide-
pulsed APCs (3 × 10^5) for the indicated times at 37°C. Reactions were
stopped by the addition of ice-cold PBS.

For analysis of Rap1, cells were lysed in 25 mM Tris (pH 7.5), 250 mM
NaCl, 1.25 mM MgCl2, 10% glycerol, 0.5% Nonidet P-40, and a mixture of
protease inhibitor (Roche). Cell nuclei were removed by centrifugation.
Clarified lysates were incubated with GST-fusion, Raf GDS-rap binding
domain (RBD) (Upstate Biotechnology) for 4°C for 1 h. The GST-Raf GDS-
RBD-bound proteins were washed and analyzed by SDS-PAGE. After
electrophoresis, the proteins were transferred onto polyvinylidene difluo-
ride membranes for immunoblotting. Blots were probed with rabbit anti-
Rap1 followed by HRP-conjugated anti-rabbit IgG. The immunoblots were
developed by ECL (Amersham Biosciences). In addition, 10% of whole
cell lysates from each sample was run to assess the total amount of Rap1
present. For analysis of Rac1 and cdc42 activation, cells were lysed in 25
mM HEPES (pH 7.5), 150 mM NaCl, 1% legal CA-630, 10 mM MgCl2,
1 mM EDTA, 10% glycerol, and a mixture of protease inhibitor. Cell
lysates were incubated with GST-fusion, PKC-θ, cdc42-binding domain
(PBD) (Upstate Biotechnology) at 4°C for 1 h. The GST-PK-C1 PBD-
bound proteins were analyzed as described above. Blots were probed with
mouse anti-Rac1 or rabbit anti-cdc42 followed by HRP-conjugated
anti-mouse IgG or HRP-conjugated anti-rabbit IgG, respectively. The total amount of Rac1 or cdc42 in the cell lysates was determined as described above.

To analyze tyrosine phosphorylation of Vav, cells were lysed in 20 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM Na3VO4, 50 mM NaF, 10 mM Na2MnO4, and a mixture of protease inhibitor. Cell extracts were incubated with protein G-Sepharose conjugated with rabbit anti-Vav. The precipitates were subjected to immunoblotting as described above, using anti-phosphotyrosine mAb 4G10 followed by HRP-conjugated anti-mouse IgG. The amount of Vav protein was determined by stripping the phosphotyrosine blot and reprobing with rabbit anti-Vav followed by HRP-conjugated anti-rabbit IgG.

Biochemical isolation of lipid raft fraction

APCs were pulsed with 50 μM OVA323–339 for 4 h at 37°C, after which they were washed three times. Splenic CD4 T cells (3 × 105) from DO11.10 mice were mixed with peptide-pulsed APCs (3 × 105) for the indicated times at 37°C. The reaction was stopped by adding ice-cold PBS. Cells were then lysed with 1 ml of lysis buffer containing 25 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Brij 98, 50 mM NaF, 1 mM Na3VO4, 10 mM Na2MnO4, and a mixture of protease inhibitor. The lysates were homogenized with 20 strokes of a Dounce homogenizer and centrifuged. Supernatants were collected and mixed with an equal volume of 80% sucrose solution and overlaid with 6.5 ml of 30% sucrose and 3.5 ml of 5% sucrose solution. The samples were subjected to ultracentrifugation in a Hitachi RPS40T rotor (Hitachi) at 35,000 rpm at 4°C for 16 h. Twelve fractions were collected from the top of the gradient. Fractions containing lipid rafts were determined using HRP-conjugated CTx. Fractions 3 and 4 or fractions 10–12 were combined and referred to as raft fractions or nonraft fractions, respectively. Proteins in raft fractions were concentrated by acetone precipitation. Pooled raft and nonraft fractions were subjected to SDS-PAGE and Western blotting with mouse anti-mouse IgG or HRP-conjugated anti-rabbit IgG.

Statistical analyses

Statistical significance was assessed by Student’s t test, with significance accepted at the p < 0.05 level.

Results

Induction of oral tolerance in DO11.10 mice

To obtain orally tolerized T cells, OVA was administered to DO11.10 TCR transgenic mice in their drinking water for 7 days. CD4 T cells were isolated from the spleens of these mice or control DO11.10 mice, which had been administered OVA-free water. First, we checked the expression levels of clonotype TCRs and naive/memory markers on CD4 T cells from OVA-fed mice. As shown in Fig. 1A, expression of KJ1.26+ TCR was slightly downregulated in CD4 T cells from OVA-fed mice. However, significant levels of KJ1.26+ TCR were expressed on these CD4 T cells. In addition, the percentage of KJ1.26+ T cells in CD4 T cells in OVA-fed mice was almost the same compared with that observed in control mice. Analysis of CD62L and CD44 expression revealed that ~60–70% of KJ1.26+ T cells showed phenotype of previously primed T cells. Therefore, it seems that oral administration of OVA to DO11.10 was effective to prime Ag-specific T cells.

Next, the functional response of splenic CD4 T cells from these mice was analyzed by measuring both proliferation and IL-2 production upon stimulation with OVA323–339 and APCs at several time points. As shown in Fig. 1, B and C, both the proliferative response and IL-2 production were significantly decreased in CD4 T cells from OVA-fed DO11.10 mice at any time point of the culture. Furthermore, in vivo OVA-specific Ab response elicited by OVA immunization in OVA-fed mice was examined. Compared with non-OVA fed control mice, anti-OVA IgG response was significantly reduced in OVA-fed mice (Fig. 1D). Consequently, we concluded that oral administration of OVA successfully induced systemic tolerance and hyporesponsiveness of Ag-specific CD4 T cells in DO11.10 mice. Hereafter, we designate CD4 T cells from non-OVA fed or OVA-fed DO11.10 mice as untreated control or orally tolerized T cells, respectively.

Orally tolerized T cells can form stable conjugates with APCs

Efficient TCR signaling following T cell activation requires conjugate formation with an APC (38). The hyporesponsive state of orally tolerized T cells may be due to inefficient conjugate formation. We first asked whether orally tolerized DO11.10 T cells could form stable conjugates with OVA-bearing APCs. T cell-depleted splenocytes from BALB/c mice were pulsed with OVA323–339 and incubated with DO11.10-splenic CD4 T cells. T cell-APC conjugates were measured using flow cytometry by staining with KJ1.26 and anti-MHC class II mAb. Interestingly, we found that orally tolerized T cells have no impairment in their formation of conjugates with APCs. Within 30 min of incubation, orally tolerized T cells formed conjugates with APCs pulsed with 1 μM OVA323–339, at comparable levels with untreated control T cells (Fig. 2A). Next, we checked the kinetics and Ag dose response of conjugate formation. In this system, conjugate formation occurred rapidly, within minutes of mixing, and reached maximal conjugate formation at ~30 min (Fig. 2B). Orally tolerized T cells formed conjugates more efficiently than untreated control T cells after 5 and 15 min of incubation (Fig. 2B). However, comparable levels of conjugate formation were seen between these T cells after 30 min. The conjugates formed by these T cells were stable for at least 3 h (data not shown). Conjugate formation was completely Ag dose dependent and no difference in Ag dose dependency was seen between untreated control and orally tolerized T cells (Fig. 2C). These results clearly show that orally tolerized T cells can respond to specific Ags quite normally in terms of forming APC conjugates.

TCR-mediated recognition of peptide/MHC complexes on APCs triggers inside-out signaling, leading to integrin activation and integrin-mediated formation of T-APC conjugates. Among a number of molecules, the β2-integrin LFA-1 plays an important role in conjugate formation (39). Therefore, LFA-1 expression on the surface of these T cells was analyzed by FACS. Orally tolerized T cells expressed slightly higher levels of LFA-1 than untreated control T cells (Fig. 3A); however, this difference had no effect on the efficiency of conjugate formation (Fig. 2). The addition of anti-LFA1 mAb to the cultures completely blocked conjugate formation by untreated control and orally tolerized T cells (Fig. 3B). These results show that orally tolerized T cells form conjugates with APCs in a LFA-1-dependent manner.

The small GTPase, Rap1, is a signaling molecule that plays a pivotal role in mediating inside-out signals to activating integrins (40). Rap1 activation by TCR stimulation enhances the adhesive activity of LFA-1 and, thus, regulates LFA-1/ICAM-1-mediated interactions between T cells and APCs (41, 42). Because orally tolerized T cells could form stable conjugates with APCs in a LFA-1-dependent manner, we thought that signaling pathways, leading to Rap1 activation, should be intact in orally tolerized T cells. We examined Ag-induced Rap1 activation in T cells by a pull-down assay using GST-RalGDS-RBD, which binds specifically to Rap1-GTP (43). As shown in Fig. 3C, antigenic stimulation significantly induced Rap1 activation in orally tolerized T cells. Rap1 activation in tolerized T cells was evident 5 min after stimulation and persisted for as long as 30 min. The levels of active Rap1 were slightly higher in orally tolerized T cells than in untreated control T cells. Thus, it was suggested that Rap1-mediated activation of LFA-1 could occur normally in orally tolerized T cells upon antigenic stimulation, resulting in efficient formation of conjugates with APCs.
Translocation of TCR, PKC-θ, and lipid rafts into the T cell-APC contact site is defective in orally tolerized T cells

It has been shown that T cells interacting with APCs accumulate Ag receptors, coreceptors, and adhesion and signaling molecules at the site of cell-cell contact, which is called the immunological synapse (23, 44). Formation of the immunological synapse is thought to be required for full activation of T cells, such as their proliferation and IL-2 production (29). We next examined whether orally tolerized T cells could accumulate signaling molecules to the contact site with APCs. Confocal microscopy was performed to investigate localization of TCR (KJ1.26) and PKC-θ at the interface between T cells and Ag-bearing APCs. T cells were mixed with APCs for 30 min because conjugate formation was predetermined optimal at this time point (Fig. 2). Fig. 4A shows a representative set of images of TCR and PKC-θ localization in both untreated control and orally tolerized T cells conjugated to peptide-pulsed APCs. We counted the number of KJ1.26 T cell-APC conjugates with translocation of TCR and PKC-θ to the contact site. Fig. 4B shows the frequencies of KJ1.26 T cell-APC conjugates with translocation of TCR and PKC-θ to the contact site. The frequency of conjugates with polarized TCR was 52.5% in untreated control T cells but was 25% in orally tolerized T cells. PKC-θ analysis showed similar results. The frequency of conjugates with polarized PKC-θ was 52.6% in untreated control T cells but was only 20% in orally tolerized T cells. These differences were statistically significant (p < 0.001 for TCR and p < 0.01 for PKC-θ). Importantly, the results indicate that immunological synapse formation was impaired in orally tolerized T cells.

It has been demonstrated that lipid rafts function as platforms for the assembly of signaling complexes and play an important role in T cell signaling (27, 45, 46). Upon antigenic stimulation, lipid rafts on T cells recruit signaling molecules and move to the contact site with APCs. To determine whether lipid raft signaling functions normally in orally tolerized T cells, we first examined lipid raft polarization to the interface between T cells and Ag-bearing APCs. Lipid rafts can be visualized by confocal microscopy with the use of fluorescently labeled CTx subunits. CTx specifically binds to the glycosphingolipid GM1, which is enriched in membrane lipid rafts (47). As can be seen in Fig. 5A, stimulation of untreated control T cells with Ag-pulsed APCs for 30 min induced translocation of lipid rafts to their contact area. However, the majority of orally tolerized T cells expressed their lipid rafts around the
plasma membrane, even if they formed conjugates with APCs. The frequency of KJ1.26⁺ T cell-APC conjugates with polarized lipid rafts was 63.2% in untreated control T cells but 22% in orally tolerized T cells. We next investigated whether signaling molecules translocate to the lipid rafts. To prepare the detergent insoluble raft fractions, cell lysates were ultracentrifuged over a sucrose step-gradient. The positions of raft and nonraft fractions in the gradient were revealed by reactivity with CTx (data not shown).

Upon antigenic stimulation, small but readily detectable increases in the amount of TCR-ζ and PKC-θ were seen in the lipid raft fractions in untreated control T cells (Fig. 5B). As expected, TCR-ζ and PKC-θ were barely detectable in lipid raft fractions in orally tolerized T cells upon stimulation with Ag (Fig. 5B). These results indicate that orally tolerized T cells have defects in their polarization of lipid rafts and translocation of signaling molecules into lipid rafts upon antigenic stimulation.

FIGURE 2. Conjugate formation of orally tolerized T cells with Ag-bearing APCs. Splenic CD4 T cells from untreated control or OVA-fed DO11.10 mice were mixed with APCs prepulsed with 1 μM OVA323–339. After 30 min, cells were harvested, fixed, and stained with anti-CD4, KJ1.26, and anti-MHC class II mAb, and the percentage of double positive cells are shown. A representative staining profile with KJ1.26 and anti-MHC class II mAb and mixed with splenic CD4 T cells from untreated control (○) or OVA-fed (●) DO11.10 mice for 30 min (B) or 5, 15, 30, and 60 min (C). The percentages of MHC class II⁺ cells within CD4⁺ KJ1.26⁺ T cell population were determined. The results are shown as the average from triplicate cultures ± SD. The results are representative of three independent experiments.

FIGURE 3. LFA-1-dependent conjugate formation by orally tolerized T cells. A. The expression levels of LFA-1 on the surface of CD4KJ1.26 cells from untreated control (thin line) or OVA-fed (thick line) DO11.10 mice were determined by FACS. The data is a representative of three independent experiments. B. APCs were prepulsed with 0.01–10 μM (B) or 1 μM (C) OVA323–339 and mixed with splenic CD4 T cells from untreated control or OVA-fed (●) DO11.10 mice for 30 min (B) or 5, 15, 30, and 60 min (C). The percentages of MHC class II⁺ cells within CD4⁺ KJ1.26⁺ T cell population were determined. The results are shown as the average from triplicate cultures ± SD. The results are representative of three independent experiments.
immunological synapse formation and raft recruitment to the synapse (48–51). Thus, we investigated tyrosine phosphorylation of Vav upon antigenic stimulation by Vav immunoprecipitation (Fig. 6A). Tyrosine phosphorylation of Vav was rapidly and significantly induced as early as 1 min upon antigenic stimulation in untreated control T cells. Phosphorylated Vav was seen 10 min after stimulation, although the levels were reduced. In sharp contrast, the levels of phosphorylation of Vav were not increased 1 min after stimulation in orally tolerized T cells. Interestingly, the levels of phosphorylation of Vav at 5 min were below those before stimulation. Phosphorylation of Vav was barely detectable 10 min after stimulation in these cells. We next investigated activation of Rac1 and cdc42 by the pull-down assay using GST-PAK-1 RBD, which specifically binds to Rac-GTP and cdc42-GTP (52). The GTP-bound, activated Rac1 and cdc42 were significantly induced upon antigenic stimulation in untreated control T cells (Fig. 6, B and C). Activation of Rac1 and cdc42 persisted for as long as 10 min. In contrast, activated Rac1 was not significantly induced beyond the levels before stimulation in orally tolerized T cells (Fig. 6B). Activated cdc42 was barely detectable upon antigenic stimulation in orally tolerized T cells (Fig. 6C). These differences in activation of Rac1 and cdc42 between untreated control and tolerized T cells were not due to differences in kinetics of activation because we did not observe increases in the levels of activated Rac1 and cdc42 at 15, 30, and 60 min after stimulation in orally tolerized T cells (data not shown). Taken together, our results indicate that defective activation of Vav/Rac1/cdc42 in orally tolerized T cells results in their defective translocation of TCR, PKC-θ, and lipid rafts into the interface between T cells and APCs.

**OVA-immunized T cells can form immunological synapses and activate vav, Rac, and cdc42**

One may argue that any Ag-experienced T cells behave differently to naive T cells in terms of immunological synapse formation. Therefore, we finally made comparisons of orally tolerized T cells vs productively primed T cells. OVA-immunized T cells were prepared from spleen of DO11.10 mice immunized i.p. with OVA. Approximately 60–80% of these T cells showed phenotypes of
memory/primed T cells as judged by CD62L and CD44 expression (data not shown). As shown in Fig. 7A, OVA-immunized T cells showed the same levels of proliferation and IL-2 production but greatly enhanced levels of IL-4 and IFN-γ production, compared with untreated control T cells, showing that these immunized T cells are productively primed to be differentiated to effector T cells. No differences were seen in conjugate formation between immunized and orally tolerized T cells (Fig. 7B). However, in contrast to orally tolerized T cells, immunized T cells could accumulate TCR, PKC-θ, and lipid rafts at the interface between T cells and APCs. The frequencies of conjugates with polarized TCR, PKC-θ, and lipid rafts in immunized T cells were higher than those of untreated control T cells (Figs. 4 and 7C). As expected, in contrast to orally tolerized T cells, Ag-induced activation of vav, Rac1, and cdc42 was seen in immunized T cells (Fig. 7D). Thus, we conclude that impaired immunological synapse formation is specifically observed in orally tolerized T cells, not in any Ag-experienced T cells.

**Discussion**

In this article, we demonstrate that orally tolerized T cells can form stable conjugates with Ag-bearing APCs. However, these cells have defects in their translocation of TCR, PKC-θ, and lipid rafts to the interface between T cells and APCs. Biochemical analysis revealed intact activation of Rap1 but defective activation of Vav,
Rac1, and cdc42 in orally tolerized T cells upon antigenic stimulation. These biochemical events induced by Ag stimulation correlate well with normal conjugate formation but impaired immunological synapse formation in orally tolerized T cells. The characteristics of TCR-mediated signaling in in vivo-tolerized T cells have been analyzed in various experimental systems (15–17). However, most of these studies used artificial, anti-TCR or anti-CD3 stimulation; therefore Ag-induced signaling, conjugate formation, and immunological synapse formation in in vivo-tolerized T cells have not been examined thus far. Therefore, this study provides new insight into Ag responsiveness of orally tolerized T cells. The findings are novel in that orally tolerized T cells are shown to form stable conjugates with APCs, without the accumulation of TCR, PKC-θ, and lipid rafts at the interface. The data suggest that not all functions are impaired in orally tolerized T cells. This is also supported by our biochemical data in that these T cells show normal activation of Rap1 (Fig. 3) and the Ras-ERK pathway (21). Furthermore, our data also suggests that conjugate formation and immunological synapse formation are regulated by distinct mechanisms. Thus, as seen in orally tolerized T cells, stable conjugate formation may not be always accompanied by immunological synapse formation.

Garcia et al. (53) have shown previously that hypersensitive T cells from aged mice have defects in their immunological synapse formation. Their results are consistent with ours in that hypersensitive T cells cannot translocate TCR or PKC-θ to the site of T-APC interaction. They also showed that various kinases, enzymes, and adaptors, such as lck, phospholipase C-γ (PLC-γ), linker for activation of T cells, Grb2, and Vav, are not accumulated in the contact site with APCs and T cells from aged mice. Recent work by Heissmeyer et al. (54) also demonstrated instability of the immunological synapse formation in in vitro-anergized T cells. These reports suggest that impaired immunological synapse formation could account for T cell hypersensitivity. In contrast, some differences were observed between our orally tolerized T cells and in vitro anergized T cells in terms of their immunological synapse formation. In vitro anergized T cells formed immunological synapses indistinguishable from those of normal T cells at early time points after incubation on lipid bilayers (54). However, the immunological synapse of anergized T cells was not stable and was broken down at later time points. We also analyzed the kinetics of accumulation of TCR and PKC-θ to the site of T-APC contact. However, we failed to observe translocation of these molecules in orally tolerized T cells at earlier time points (data not shown). The differences in synapse formation at early time points may be derived from the differences in the experimental systems, such as in vitro vs in vivo hypersensitive T cells as responders, or lipid bilayers or live APCs as stimulators. It is important, anyhow, that in both studies, the majority of hypersensitive T cells did not form proper immunological synapses at later time points because prolonged immunological synapse formation appears to be required for full T cell activation (29).

We demonstrated that translocation of lipid rafts to the contact site and TCR/PKC-θ recruitment to the lipid rafts is defective in orally tolerized T cells upon antigenic stimulation (Fig. 5). Lipid rafts are plasma membrane compartments enriched in key signaling molecules, such as lck, fyn, and linker for activation of T cells, and are thought to function as platforms in TCR signaling. Aggregation and translocation of lipid rafts is thought to stabilize the immunological synapse and result in sustained signaling leading to full activation (55). Thus, the failure of lipid raft clustering and translocation of signaling molecules into lipid rafts may result in defects in immunological synapse formation and affect downstream signaling events. It has been demonstrated that translocation of PKC-θ into lipid rafts and immunological synapse is required for NF-κB activation in T cells (28). Consistent with this, we have found that NF-κB nuclear translocation is defective in Ag-stimulated orally tolerized T cells (unpublished observation).

Orally tolerized T cells showed defective activation of Vav, Rac1, and cdc42 upon antigenic stimulation (Fig. 6). Knockout studies revealed that Vav is a critical regulator for rearrangement of the actin cytoskeleton, TCR capping, and cell adhesion (48, 51). Furthermore, it has been shown that lipid raft polarization into the immunological synapse depends on Vav/Rac function (50). Wiskott-Aldrich syndrome protein, a cdc42 effector, has also been shown to be required for TCR capping, lipid raft clustering, and immunological synapse formation (56). Taken together, defective activation of Vav/Rac1/cdc42 in orally tolerized T cells may be responsible for their impaired immunological synapse formation and raft translocation to the synapse.

The molecular basis underlying defective Vav activation in tolerized T cells remains unclear. However, we noted unique patterns of tyrosine phosphorylation of Vav observed in orally tolerized T cells upon antigenic stimulation (Fig. 6A). The levels of tyrosine phosphorylation of Vav 5 min after stimulation was below those of unstimulated T cells, although the levels of Vav protein were not changed. This may be explained by the idea that negative regulators for Vav are strongly activated upon stimulation and such molecules induce down-regulation of Vav phosphorylation in orally tolerized T cells. One of candidate negative regulator may be cbl-b, an E3 ubiquitin ligase (57, 58). Vav phosphorylation is strongly up-regulated in cbl-b−/− T cells upon TCR stimulation, suggesting that cbl-b is a negative regulator of Vav phosphorylation (59, 60). We found that cbl-b is highly expressed in orally tolerized T cells (data not shown). We are now investigating the mechanism of Ag-induced down-regulation of Vav phosphorylation in orally tolerized T cells, including the involvement of cbl-b activation.

We found that Rap1 activation remains intact in orally tolerized T cells upon antigenic stimulation (Fig. 3). Rap1 is a potent activator of integrins and is essential for LFA-1/ICAM-1-mediated interactions between T cells and APCs (41, 42). Inhibition of Rap1 activation by a dominant-negative Rap1 abrogates T-APC conjugate formation (41). Conversely, overexpression of Rap1 enhances conjugate formation (41). Thus, normal conjugate formation by orally tolerized T cells seems to be due to intact activation of Rap1.

Katagiri et al. (61) have demonstrated recently that Rap1 activation induced by TCR stimulation is dependent on PLC-γ and that Rap1 activation is likely to be mediated by CalDAG-GEFI. In contrast, Amsen et al. (62) reported that Rap1 activation in thymocytes is induced in a PLC-γ-independent manner. The study also suggested that Cbl-C3G-CrkL may play an important role for Rap1 activation in thymocytes. We have demonstrated that PLC-γ is not activated in orally tolerized T cells upon TCR stimulation (21). Thus, it is likely that Rap1 activation in orally tolerized T cells is induced independently of PLC-γ pathways, as in the case of thymocytes.

It has been reported that human anergic T cell clones express active Rap1 constitutively (63) and that overexpression of constitutively active form of Rap1 inhibits IL-2 production by T cells by the down-regulation of Ras-ERK activation (63). The function of Rap1 as a negative regulator for T cell activation was confirmed by a knockout study of SPA-1, a principle Rap1 GTPase-activating protein, showing that accumulation of large amounts of active Rap1 correlated with T cell hypersensitivity and down-regulation of ERK activation (64). In our system, the levels of active Rap1 in orally tolerized T cells was higher, but only slightly, than those in control T cells (Fig. 3). Furthermore, Ras-ERK activation
induced by TCR stimulation was normal in orally tolerized T cells (21). Thus, it seems unlikely that Rap1 activation contributes to the hyporesponsiveness of orally tolerized T cells.

The biological meaning of normal conjugate formation by orally tolerized T cells is currently unknown. However, the formation of stable conjugates with APCs may help to regulate their own activation. It may be possible that stable and prolonged conjugate formation enables these T cells to elicit negative signaling, such as ubiquitin-mediated degradation of signaling proteins or phosphatase activation or to cause their own apoptosis. It has been demonstrated that orally tolerized T cells have immunoregulatory functions via the production of suppressive cytokines such as IL-10 or TGF-β (6, 65). Stable conjugate formation may be required to elicit such effector functions by tolerized T cells. These points should be clarified in future studies.

One may argue that regulatory T cells may play a role in our OVA-induced tolerance system. CD4 T cells from OVA-fed DO11.10 mice contained 15–30% of CD25+ T cells (data not shown). Consistent with recent reports suggesting the suppressive function of CD4CD25+ T cells in oral tolerance (66, 67), CD4CD25+ T cells in OVA-fed DO11.10 mice were unresponsive to TCR stimulation but were able to inhibit naïve T cell activation (data not shown). It is unknown whether these CD4CD25+ T cells play any roles in induction of oral tolerance. However, depletion of the CD4CD25+ T cells did not affect the proliferative response and IL-2 production by CD4 T cells from OVA-fed mice (data not shown), suggesting that the hyporesponsiveness of CD4 T cells in our system was not mediated by the CD4CD25+ T cells. So far a few studies have addressed TCR-mediated signaling in naturally occurring CD4CD25+ T cells (68, 69), but their characteristics concerning immunological synapse formation is still unknown. The comparison between anergic and CD4CD25+ regulatory T cells in terms of their TCR-mediated signaling and immunological synapse formation should be performed.

In conclusion, this study demonstrates that orally tolerized T cells can form conjugates with APCs efficiently but fail to translocate TCR, PKC-θ, and lipid rafts to the contact site. This impairment of immunological synapse formation may be responsible for the hyporesponsiveness of orally tolerized T cells. These findings will be helpful in providing a deeper understanding of the molecular mechanisms of peripheral tolerance.

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Disclosures

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References


