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Seeyoung Choi and Ronald H. Schwartz

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Adaptive tolerance is a hyporesponsive state in which lymphocyte Ag receptor signaling becomes desensitized after prolonged in vivo encounter with Ag. The molecular mechanisms underlying this hyporesponsive state in T cells are not fully understood, although a major signaling block has been shown to be present at the level of ZAP70 phosphorylation of linker for activation of T cells (LAT). In this study, we investigated the ability of adaptively tolerant mouse T cells to form conjugates with Ag-bearing APCs and to translocate signaling molecules into the interface between the T cells and APCs. Compared with naive or preactivated T cells, adaptively tolerant T cells showed no dramatic impairment in their formation of conjugates with APCs. In contrast, there was a large impairment in immunological synapse formation. Adaptively tolerant T cells were defective in their translocation of signaling molecules, such as ZAP70, LAT, and phospholipase Cγ1, into the T cell–APC contact sites. Although Ag-induced activation of VAV1 was normal, VAV’s recruitment into the synapse was also impaired. Interestingly, expressions of both IL-2–inducible T cell kinase and growth factor receptor-bound protein 2-related adaptor downstream of SHC were decreased by 60–80% in adaptively tolerant T cells. These decreases, in addition to the impairment in LAT phosphorylation by ZAP70, appear to be the major impediments to the phosphorylation of SLP76 (SRC homology 2 domain-containing leukocyte protein of 76 kDa) and the recruitment of VAV1, which are important for stable immunological synapse formation. The Journal of Immunology, 2011, 187: 000–000.

Laboratory of Cellular and Molecular Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

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Address correspondence and reprint requests to Dr. Ronald H. Schwartz, Laboratory of Cellular and Molecular Immunology, Department of Health and Human Services, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Building 4, Room 111, MSC-0420, Bethesda, MD 20892-0420. E-mail address: r334t@niaid.nih.gov

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Abbreviations used in this article: ADAP, adhesion- and degranulation-adaptor protein; CTx, cholera toxin; DAG, diacylglycerol; GADS, growth factor receptor-bound protein 2-related adaptor downstream of SHC; GSK, glycogen synthase kinase; ITK, IL-2–inducible T cell kinase; LAT, linker for activation of T cells; LN, lymph node; MCC, moth cytochrome c; MTOC, microtubule-organizing center; NP40, Nonidet P-40; PBD, p21 binding domain; PCC, pigeon cytochrome c; PRC8, protein kinase Cθ; PLCγ1, phospholipase Cγ1; RBD, RAP binding domain; SLP76, SRC homology 2 domain-containing leukocyte protein of 76 kDa; SMAC, supramolecular activation cluster.

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After initial conjugate formation, the TCRs and signaling molecules are recruited to the interface between the T cell and the APC (29–31), where they arrange into a complex known as the central supramolecular activation cluster (SMAC) (29). The β2 integrins also accumulate at the immune synapse in a ring surrounding the TCR signaling core (peripheral SMAC) (29). The purpose of this overall structure is still not clearly defined.Initially, it was thought to be the main mechanism for sustained signaling of the T cell, to ensure full downstream activation of the transcription factors required for differentiation and cell cycle entry. Some more recent studies, however, have suggested that its main function may be to terminate the signaling of TCR microclusters (32, 33). Nonetheless, a failure to form these synapses is usually correlated with a reduced or abortive T cell activation process (34, 35).

The VAV family of proteins plays a key role in stabilizing the immunological synapse. They do this in two different ways (36, 37). One is through their contribution to the assembly of the TCR signaling complex involving LAT, SRC homology 2-domain-containing leukocyte protein of 76 kDa (SLP76), IL-2–inducible T cell kinase (ITK), and PLCγ1. Without VAV1 present, this complex is not stably formed and downstream activation of calcium flux and ERK (early response kinase) activation are both impaired (38). Second is by recruiting molecules such as HSP (hematopoietic cell-specific protein-1) (39), DNMI2 (dynamin-2) (40), NCK (noncatalytic region of tyrosine kinase) (41), and AB1/2 (abelson-interacting protein 1 or 2) (42) to help activate the small Rho GTPases such as RAC1 and CDC42, which, in turn, help to catalyze the polymerization of actin filaments (F-actin) at the contact site by facilitating the proteins WAVE2 (WASP-family verprolin-homologous protein-2) (43) and WASP (Wiskott–Aldrich syndrome protein) (41) to activate ARP2/3 (actin-related proteins 2 and 3) (41). VAVs must first be activated by tyrosine phosphorylation after TCR engagement to function (36). This phosphorylation is mediated by SRC-family kinases (44, 45) and can be enhanced by costimulation through the CD28 receptor (46). Mice deficient in VAV-family proteins show major defects in both T cell responses and development (47). Polymerization of F-actin is also accompanied by polarization of the Golgi apparatus and the T cell microtubule-organizing center (MTOC) toward the APC (48, 49). The molecular pathways that are directly involved in the movement of the MTOC after TCR engagement are poorly defined, but a role for the motor protein dynein interacting with ADAP has been implicated (50).

Our previous studies using Abs against TCR, CD3, CD4, and CD28 (6) demonstrated that signaling through the TCR in adaptively tolerant T cells is impaired. In these experiments, we explored in detail the response of these anergic T cells to TCR stimulation by Ag and APCs. Although initial conjugate formation appeared to be normal, we found that immunological synapse formation was greatly reduced. This led to the discovery that ITK and growth factor receptor-bound protein 2-related adaptor downstream of SHC (GADS) molecules were downregulated in these cells, and that this, coupled with our previous finding of impairment in LAT phosphorylation, resulted in a failure to recruit VAV proteins into the T cell/APC interface. This, in turn, impaired actin filaments from congregating at the synapse and prevented the MTOC from orienting toward this site.

**Materials and Methods**

**Mice and adoptive transfer experiments**

All the mice used in this study were bred at the National Institute of Allergy and Infectious Diseases contract facility at Taconic Farms (Germantown, NY). They were on the B10.A (H-2b) background. For adoptive transfer experiments, the donor T cells were from B10.A, TCR-5C.C7 transgenic, Rag2−/− mice that specifically recognize the pigeon cytochrome c (PCC) peptide 81–104 bound to I-Ek (6). The recipient mice (RO) were transgenic for PCC expression under the control of an MHC class I promoter and an Ig enhancer (6). They were also CD3ε−/−. Three million lymph node (LN) T cells from TCR-5C.C7 transgenic mice were injected i.v. into each recipient mouse. Mice were sacrificed from 4–6 wk after transfer to provide the adaptively tolerant T cells. All of the animals were maintained in a specific pathogen-free environment, and all experiments were approved by the National Institutes of Health Animal Care and Use Committee.

**Abs and reagents**

Moth cytochrome c (MCC) peptide (aa 88–103 of MCC) was synthesized through the National Institute of Allergy and Infectious Diseases Peptide Facility (National Institutes of Health, Bethesda, MD). The P13K inhibitor LY294002 was purchased from Sigma-Aldrich. The SRC-family kinase inhibitor PP2 and a control analog PP3 that only inhibits the epidermal growth factor receptor kinase were purchased from Calbiochem. The following Abs were used for flow cytometry: anti-CD4-PE-Cy5.5 (Caltag Laboratories) and anti-β2-3-PE, anti-MHC class II (I-Ek)-PE, anti-CD4-biotin, anti-CD80, and ICAM1, and were kindly provided by Dr. R.N. Germain (National Institute of Allergy and Infectious Diseases, National Institutes of Health). P13.9 cells in log phase were pulsed with various concentrations of MCC peptide for 2 h in fresh medium at 37°C.

**In vitro preactivated cells**

In vitro preactivated TCR-SC.C7 transgenic cells were made by stimulating naive LN and splenic T cells with 1 μM MCC and a 10-fold excess of irradiated (3000 rad) B10.A, CD3ε−/− splenic APCs in Eagles Hank’s amino acids/RPMI 1640 medium (6). After 72 h, the activated T cells were expanded with 10 U/ml IL-2 (BioSource). The cells were then rested in fresh medium without IL-2 and used after 10 d.

**Flow cytometry**

Cell suspensions were stained with anti-β2-3-PE, anti-CD4-PE-Cy5.5. For analysis of CD62L, CD44, and LFA-1 expression, T cells were stained with anti-CD62L-FITC, anti-CD44-FITC, or anti-LFA-1–FITC (BD Pharmingen). Abs used for immunophenotyping and Western blot were from Upstate Biotechnology (anti-LAT, anti-PLCγ1, and anti-phosphotyrosine [4G10]-biotin), Santa Cruz Biotechnology (anti-VAV [sc132] and anti-protein kinase c θ [sc320], anti-CD80, and anti-BD Pharmingen (anti-TCR-β, anti-CD4-biotin, anti-CD3-biotin, anti-CD28-biotin, anti-CD62L-FITC, and anti-LFA-1–FITC). Abs used for immunoprecipitation and Western blot were from Upstate Biotechnology (anti-LAT, anti-phosphotyrosine 191 LAT, anti-VAV, anti-GADS, and anti-SLP76), Cell Signaling Technology (anti-phospho-AKT mAb, and anti-phospho-glycogen synthase kinase (GSK3)/β, Ab), Santa Cruz Biotechnology (anti-ZAP70 [sc574] anti-CD42 [sc87], anti-VAV [sc132], and anti-RAP1 [sc55]), BD Pharmingen (anti-RAC1, anti-ITK, anti-ZAP70, and anti-phosphotyrosine 319 ZAP70), Sigma-Aldrich (anti-actin), or Bio-Rad (anti-mouse IgG-HRP and anti-rabbit IgG-HRP). Streptavidin was purchased from Southern Biotechnology.

**Cell preparation**

The CD4+ T cell population was purified (>90%) from LN and spleen cells by negative selection as previously described (51). P13.9 cells, used as APCs, are fibroblasts that had been transfected with MHC class II I-Ek, CD3, and ICAM1, and were kindly provided by Dr. R.N. Germain (National Institute of Allergy and Infectious Diseases, National Institutes of Health). P13.9 cells in log phase were pulsed with various concentrations of MCC peptide for 2 h in fresh medium at 37°C.

**In vitro preactivated cells**

In vitro preactivated TCR-SC.C7 transgenic cells were made by stimulating naive LN and splenic T cells with 1 μM MCC and a 10-fold excess of irradiated (3000 rad) B10.A, CD3ε−/− splenic APCs in Eagles Hank’s amino acids/RPMI 1640 medium (6). After 72 h, the activated T cells were expanded with 10 U/ml IL-2 (BioSource). The cells were then rested in fresh medium without IL-2 and used after 10 d.
anti-4E1-PE and analyzed by flow cytometry. Conjugates were identified as the percentage of CD4+ cells that were also MHC class II *I-E*).

**Immunocytochemistry**

P13.9 APCs were pulsed with MCC for 2 h. Purified naive, tolerant, and preactivated T cells (2 × 10^5) were mixed with the APCs (4 × 10^4) at a 1:2 ratio. The cell mixtures were incubated at 37°C for 5 or 20 min and then gently resuspended and spread (50 μl/slide) onto prewarmed poly-L-lysine–coated slides (Sigma-Aldrich). Slides were incubated for another 15 min at 37°C to promote cell attachment. For lipid raft staining, CD4+ T cells were stained with FITC-CTx before incubation with the APCs. Cells were fixed with 4% paraformaldehyde in PBS for 10 min and then permeabilized with 0.2% Triton X-100 for 5 min. Cells were blocked overnight with 10% horse serum/1% BSA/PBS and stained with anti-TCRβ-biotin or anti-phosphotyrosine (4G10)-biotin followed by Alexa Fluor 488-streptavidin. For LFA-1, cells were stained with Alexa Fluor 488/anti-LFA-1. For ZAP70, cells were stained with anti–ZAP70-FITC. For LAT, PLCγ1, VAV, or PKCδ, cells were stained with rabbit anti–LAT, mouse anti–PLCγ1, rabbit anti-VAV, or rabbit anti–PKCδ, followed by donkey anti-mouse IgG-FITC or donkey anti-rabbit IgG-FITC. All images were taken using a Leica SP1 laser-scanning confocal microscope. Twenty-five to 50 conjugates were scored per condition. Data were analyzed by a two-way ANOVA, and a Bonferroni multiple-comparison test was used to determine statistical significance, which was accepted at a p value <0.05 (GraphPad Prism software). A χ^2 test was used to analyze the significance of the data on actin polymerization, MTOC polarization, and V AV translocation.

**Results**

Adaptively tolerant CD4+ T cells can form conjugates with splenic APCs

Naive PCC-specific TCR-transgenic CD4+ T cells express high levels of CD62L and low to intermediate levels of CD44 (Fig. 1A). Ten days after preactivation with Ag and splenic APCs, 55% of the T cells expressed high levels of CD44 and 67% expressed low levels of CD62L. Adaptively tolerant T cells, representing naïve TCR transgenic cells injected into DC3e–/– hosts expressing the Ag and isolated several weeks later (see Materials and Methods), had a phenotype more like that of the T cells preactivated in vitro. Two thirds of them expressed high levels of CD44, and 95% expressed low levels of CD62L (Fig. 1A). Despite their activated phenotype, however, the tolerant T cells failed to produce much IL-2 when stimulated with agonist peptide and splenic APCs (see Ref. 6).

Efficient TCR signaling for normal T cell activation requires conjugation formation with an APC. The hyporesponsive state of adaptively tolerant T cells could thus be due to inefficient conjugate formation. To investigate whether tolerant T cells could form conjugates with APCs, we purified the CD4+ transgenic T cells and mixed them with spleen cells from CD3e–/– mice, with or without the Ag MCC peptide 88–103. After fixation and flow cytometry analysis, few conjugates were detected in the absence of Ag, no matter which cell population was examined: naive, preactivated, or tolerant T cells (Fig. 1B). Within 5 min, however, a significant number of conjugates were detected (Fig. 1C). Tolerant cells formed as many conjugates as naive T cells, but less than that achieved with the preactivated T cells. The maximum numbers were achieved around 30–60 min when 1 μM MCC peptide was used (Fig. 1C).

TCR-mediated recognition of peptide/MHC complexes on APCs triggers inside-out signaling, leading to integrin activation and integrin-mediated formation of T cell/APC conjugates. Among a number of molecules, the β2 integrin LFA-1 plays an important role in this conjugate formation. Therefore, LFA-1 expression on the surface of these T cells was analyzed by FACS. Tolerant and preactivated T cells expressed 3-fold higher levels of LFA-1 than naive T cells (Fig. 1D). The addition of anti–LFA-1 mAb to the cultures nearly completely blocked all conjugate formation in the presence of Ag by naive, tolerant, and preactivated T cells (Fig. 1E). In contrast, anti-CD80 had no effect. The pretreatment with anti-CD80 also had no effect when the T cells were stimulated with lower concentrations of the peptide (Supplemental Fig. 1). These results show that LFA-1/ICAM interactions are the essential component for conjugate formation by all the T cell populations under these circumstances.
The small GTPase, RAP1, is a signaling molecule that plays a critical role in mediating inside-out signaling to activate β2 integrins. Because the tolerant T cells showed relatively stable LFA-1-dependent conjugates with splenic APCs, we thought that the signaling pathways leading to RAP1 activation would be intact in tolerant T cells. To test this, we examined TCR-stimulated RAP1 activation with a pull-down assay using GST-RAL-RBD, which binds specifically to the RAP1-GTP active form. Naive, resting T cells had very low levels of RAP1-GTP, but Ab activation with anti-CD3 and anti-CD28 (Fig. 2A) or anti-TCR and anti-CD4 (Fig. 2B) induced a substantial increase within 1–2 min. Preactivated, resting T cells, in contrast, already had increased levels of RAP1-GTP, although Ab stimulation increased this level further. The adaptively tolerant T cells also showed a significant level of RAP1-GTP in the resting state, but less than that seen in the preactivated, resting T cells. The increase in RAP1-GTP after Ab stimulation, however, was very modest, resulting in levels that were below either the naive or preactivated, stimulated T cells. Nonetheless, we conclude that the signaling pathways leading to RAP1 activation would be intact in tolerant T cells. To circumvent this problem, we switched to a fibroblast cell line called P13.9 as the APC. This cell line can present the MCC agonist peptide because P13.9 has been transfected with the MHC class II molecule, I-Ek, as well as the costimulatory molecule CD80, and the cell adhesion molecule ICAM1. As shown in Fig. 3A, P13.9 APCs form conjugates with a number of conjugates under maximal activation conditions (Fig. 1B, 1C) using the adaptively tolerant T cells, despite their increased level of LFA-1 expression. Nonetheless, we conclude that the adaptively tolerant T cells are capable of forming conjugates to a significant degree.

Adaptively tolerant CD4⁺ T cells form conjugates with P13.9 APCs

Our initial plan was to next look at the T cell/APC interface for formation of an immunological synapse in tolerant cells. However, we could not obtain very good fluorescent signals with the control naive or preactivated T cells when we used splenic APCs. To adaptively tolerant, or preactivated T cells for 30 min (B) or pulsed with 1 μM MCC(88–103) peptide and mixed for 5, 30, or 60 min (C). The cells were harvested, fixed, and stained with anti-CD4 and anti-MHC class II. The percentages of MHC class II⁺ cells within the CD4⁺ T cell population were determined. B, Data are averages from two experiments. C, Data are all from one experiment. D, The expression levels of LFA-1 on the surface of CD4⁺ and Vβ³⁺ cells from purified naive (purple), adaptively tolerant (blue line), or preactivated T cells (red line) were determined by FACS. E, The splenocytes from B10.A CD3ε⁻/⁻ mice were prepulsed in the presence or absence of 1 μM MCC(88–103) peptide and mixed with purified naive, adaptively tolerant, or preactivated T cells for 30 min in the presence or absence of anti-LFA-1 mAb (1 μg/2 × 10⁵ cells) or anti-CD80 mAb (2 μg/2 × 10⁵ cells). The percentages of CD4⁺ and MHC class II⁺ conjugates were determined. Results shown are the means ± SD for three experiments.

FIGURE 1. Phenotype and conjugate formation of adaptively tolerant T cells with spleen cells. A, Purified naive, adaptively tolerant, or preactivated 5C.C7 T cells were stained with anti-CD4, anti-Vβ3, and anti-CD62L or anti-CD44 mAb. Expression of CD62L and CD44 on Vβ³⁺CD4⁺ T cells is shown. Percentages of CD62Lhigh and CD44high T cells are indicated. B and C, Splenocytes from B10.A CD3ε⁻/⁻ mice were prepulsed with 0, 0.01, 0.1, or 1 μM MCC(88–103) peptide and mixed with naive (●), tolerant (○), or preactivated (△) T cells for 30 min (B) or pulsed with 1 μM MCC(88–103) peptide and mixed for 5, 30, or 60 min (C). The cells were harvested, fixed, and stained with anti-CD62L or anti-CD44 mAb. Expression of CD62L and CD44 on Vβ³⁺CD4⁺ T cells is shown. Percen-

FIGURE 2. RAP1 activation in adaptively tolerant T cells. A and B, Purified naive, adaptively tolerant, or preactivated T cells were unstimulated or stimulated for 2 or 5 min with anti-CD3-biotin mAb (1 μg/ml) and anti-CD28-biotin mAb (5 μg/ml) (A) or for 1 min with anti-TCRβ-biotin mAb (1 μg/ml) and anti-CD4-biotin mAb (10 μg/ml) (B) after cross-linking with streptavidin. Samples were lysed with 1% NP40 buffer, and active GTP-bound RAP1 was detected with a pull-down assay using GST-fusion RAL GDS-RBD. Bound RAP1 (upper gels) and total RAP1 (lower gels) were detected by Western blotting with anti-RAP1 Ab. The density of each band was determined using GelPro software. The relative values normalized to the total level of RAP1 expression are shown in the lower graphs. Data show one representative experiment from two that were performed.
could stimulate naive and preactivated T cells to produce IL-2 in a peptide dose-dependent manner, peaking at 1 μM peptide (Fig. 3A). In contrast, the amount of IL-2 produced by the tolerant T cell population was 10% of this maximal amount. These results are similar to our previous experiments carried out with splenic APCs (6), and thus allowed us to use P13.9 as the APC for our conjugate and synapse formation experiments.

When P13.9 cells were used in the conjugate assay, the patterns observed with naive T cells were similar to those observed with splenic APCs. Less than 10% of naive T cells formed conjugates in the absence of Ag (Fig. 3B, 3D). Thirty minutes after stimulation with 1 μM MCC peptide, however, >50% of these cells were involved in conjugates. The number of conjugates formed depended on the dose of the Ag (Fig. 3B), and their formation was detected as early as 5 min after mixing the cells (Fig. 3C). Maximal conjugate formation was achieved with 1 μM peptide after 30 min of incubation (Fig. 3C). In contrast, many preactivated T cells (48 ± 4%) were able to form conjugates in the absence of Ag. Addition of MCC peptide increased the number of conjugates by ~1.5-fold, and this was Ag dose dependent (Fig. 3B). The maximum number formed was achieved at 30 min with a dose of 1 μM peptide (Fig. 3C). The number of conjugates formed by the adaptively tolerant T cells in the absence of Ag was also quite significant (27 ± 6%), but it was intermediate between that of the naive and preactivated T cell populations. In the presence of 1 μM peptide for 30 min, this increased to 54 ± 3% (Fig. 3B, 3D). Thus, similar to the results with splenic APCs, the tolerant T cells can form conjugates with P13.9 APCs, but only half of these required Ag stimulation to initiate the process.

The significant number of conjugates observed in the absence of Ag with P13.9 APCs appears to be at least partially caused by the high expression of CD80 on these cells. Such conjugates could be significantly inhibited with anti-CD80 mAb (Fig. 3D). A similar Ab-mediated inhibition was seen with anti–LFA-1 (Fig. 3D), suggesting that both molecules are required. The anti-CD80 inhibition was less prominent after peptide stimulation, but still significant for the naive and tolerant T cells. LFA-1, in contrast, was nearly completely inhibitory under all conditions (Fig. 3D).

The reason that Ag-independent conjugates are seen only with the preactivated and tolerant T cells could be because they express 3-fold higher levels of surface LFA-1 (Fig. 1D). Imaging of the T cell/APC interface at 5 or 20 min after formation of the conjugates showed an enrichment of LFA-1 at the interface (compared with the rest of the plasma membrane) for all three T cell populations (Supplemental Fig. 2). The ratio of the mean signal intensity at the interface relative to the intensity over the rest of the T cell membrane was not significantly different for the preactivated and adaptively tolerant T cells, which express equally high levels of LFA-1. In contrast, the naive cells showed a somewhat larger ratio, because the total signal in these cells was lower as a consequence of their decreased level of LFA-1 expression in the membrane.

**Translocation of signaling molecules into the contact site is impaired in adaptively tolerant T cells**

Immunological synapse formation is required for full activation of T cells leading to consequences such as proliferation and IL-2 production. We investigated whether adaptively tolerant T cells could accumulate signaling molecules in their contact site with the APCs, which is the hallmark of this process. Purified T cells were initially incubated with MCC peptide-pulsed P13.9 APCs, and confocal microscopy was used to see the localization of ZAP70, LAT, PLCγ1, and PKCθ at the T cell/APC interface. T cells were mixed with the APCs for 5 min because the extent of conjugate formation was similar at this time point in all three populations examined: naive, preactivated, and tolerant T cells (Fig. 3C). For these four proteins involved in early TCR signal transduction, both naive and preactivated T cells showed a polarized pattern of localization at the T cell/APC interface as measured by the ratio of the inside-to-outside distribution of Ab staining at the synapse.
In contrast, no polarization was observed for these proteins in the tolerant T cells, although the enhanced expression of ZAP70 in the tolerant T cells (6) (Fig. 5A) made this particular analysis more difficult. Nonetheless, the difference from the naive or preactivated T cells was statistically significant for the means of 25 measurements in each of two experiments. We conclude that the translocation of ZAP70, LAT, PLC\(_\gamma1\), and PKC\(_\mu\) into the T cell/APC contact area after Ag stimulation is impaired in the tolerant T cells.

TCR clustering in the immunological synapse is another polarization event that accompanies T cell activation by Ag and APC. As shown in Fig. 6A, this was observed for naive and preactivated T cells, but not for the tolerant T cells. TCR engagement also leads to the phosphorylation of many proteins on tyrosine residues. This can be detected with an anti-phosphotyrosine mAb. In naive and preactivated T cells, the distribution of these phosphorylated proteins was preferentially polarized over the T cell/APC synapse. In contrast, for the tolerant T cells, no significant polarization was seen. These differences from the naive and preactivated T cells were significant. Finally, lipid raft polarization to the T cell/APC contact site was measured using CTx. CTx specifically binds to the glycosphingolipid GM1, which is enriched in membrane lipid rafts. As shown in Fig. 6C, GM1 accumulated in the T cell/APC interface after Ag-induced activation of both naive and preactivated T cells, but not at the interfaces of tolerant T cells. This difference was also statistically significant (p < 0.001). Overall, these observations demonstrate that the formation of an immunological synapse after Ag-induced activation is greatly impaired in the adaptively tolerant T cells.

**FIGURE 4.** Translocation of signaling molecules to the T cell/APC contact region is greatly impaired in adaptively tolerant T cells. A–D, Purified naive, adaptively tolerant, or preactivated T cells were stimulated for 5 min with the P13.9 cell line prepulsed with 1 \(\mu\)M MCC(88–103). Cells were fixed, permeabilized, and stained for PKC\(_\mu\), PLC\(_\gamma1\), LAT, or ZAP70. Representative images for T cell/APC conjugates and the localization of PKC\(_\mu\), PLC\(_\gamma1\), LAT, or ZAP70 are shown in the left panels. The localization of PKC\(_\mu\), PLC\(_\gamma1\), LAT, or ZAP70 was measured with ImageJ software (http://rsb.info.nih.gov/ij/). Means ± SEM of the inside to outside distribution of staining at the synapse for (A) PKC\(_\mu\), (B) PLC\(_\gamma1\), (C) LAT, or (D) ZAP70 are shown in the right panels. Twenty-five conjugates were counted in each of two experiments. Original magnification \(\times63\). *p < 0.05, **p < 0.01, ***p < 0.001.

**Activation of ZAP70 and LAT, but not activation of VAV1, RAC1, and CDC42, is impaired in adaptively tolerant T cells**

Next, we analyzed the biochemical basis for the defective translocation of signaling molecules to the T cell/APC interface of tolerant T cells. ZAP70 activity is required for the recruitment of signaling molecules such as PKC\(_\mu\) and PLC\(_\gamma1\) to the synapse (52). To examine its activation, we stimulated naive and tolerant CD4\(^+\) T cells with Ag and P13.9 APCs for 2 or 5 min. As we reported previously with splenic APCs (6), ZAP70 phosphorylation was decreased in tolerant T cells compared with naive T cells, when normalized to the total amount of ZAP70 immunoprecipitated (Fig. 5A). Part of this effect, however, was caused by an increase in the level of ZAP70 in the tolerant T cells only. Tyrosine phosphorylation of the scaffolding molecule LAT was also decreased in the tolerant T cells (Fig. 5A). This dramatic effect, however, was not impacted by any change in the level of the protein. Thus, a major block in TCR signaling in tolerant T cells responding to peptide/MHC is at the level of ZAP70 phosphorylation of LAT.
VAV, a guanine nucleotide exchange factor for the Rho-family GTPases RAC1 and CDC42, is a critical regulator of immunological synapse formation. We investigated its tyrosine phosphorylation in anti-VAV1 immunoprecipitates after Ag/APC stimulation. Interestingly, VAV1 phosphorylation was not decreased in the tolerant T cells compared with naive T cells (Fig. 5A). In fact, its phosphorylation was not reduced in either cell type out to 20 min (Supplemental Fig. 3B). We also stimulated these cells with anti-TCRβ and anti-CD4, and obtained similar results (Fig. 5B, left panels). When the cells were stimulated with anti-TCRβ and anti-CD28, the tolerant T cells actually showed a slightly increased VAV phosphorylation compared with that of naive T cells, although it was similar to the amount seen in stimulated preactivated T cells (Fig. 5B, right panels).

We also investigated activation of the downstream GTPases RAC1 and CDC42 by a pull-down assay using GST-PAK1-PBD, which specifically binds to RAC-GTP and CDC42-GTP. When the cells were stimulated with anti-TCRβ and anti-CD4 or anti-CD3 and anti-CD28, tolerant T cells showed an intact activation of RAC1 and CDC42 (Fig. 5C), equivalent to that seen in preactivated T cells.

F-actin enrichment and MTOC polarization at the immune synapse are impaired in adaptively tolerant T cells

Because tolerant T cells could efficiently form conjugates with APCs and VAV1 signaling seemed to be intact, we wondered whether the enhanced actin polymerization that normally occurs at the immunological synapse would also take place. However, tolerant T cells did not accumulate augmented amounts of F-actin (phalloidin staining) at the synapse 20 min after peptide/MHC presentation by P13.9 APCs (Fig. 7A). In contrast, naive and preactivated T cells do show this enhancement. Moreover, labeling with anti-tubulin revealed that the tolerant T cells do not polarize their MTOC toward the bound APCs as efficiently as either naive or preactivated T cells (Fig. 7B). A χ² statistical analysis of two experiments showed that the tolerant T cells have impairments in both of these Ag-induced responses.

VAV synapse localization is impaired in adaptively tolerant T cells

In TCR-induced T cell activation, VAV plays a major role in the initiation of actin polymerization during the formation of the immunological synapse.
FIGURE 6. Translocation of the TCR, tyrosine-phosphorylated proteins, and lipid rafts to the T cell/APC contact site is impaired in adaptively tolerant T cells. A and B, Purified naive, adaptively tolerant, or preactivated T cells were stimulated for 5 min with the P13.9 cell line prepulsed with 1 μM MCC (88–103). Cells were fixed, permeabilized, and stained for TCRs or tyrosine-phosphorylated molecules. Representative images for T cell/APC conjugates and the localization of TCRs or tyrosine-phosphorylated molecules are shown (left panels). The ratios of the mean number of phosphorylated molecules or TCRs in/out of the synapse measured with ImageJ software are shown (right panels). *p < 0.05, **p < 0.01, ***p < 0.001. C, Purified naive, adaptively tolerant, or preactivated T cells were stained with FITC-CTx and then incubated with the P13.9 cell line prepulsed with 1 μM MCC (88–103). After 5 min, the cells were fixed. Representative images for T cell/APC conjugates and localization of the lipid rafts are shown (left panels). The mean CTx ratios in/out of the synapse were measured with ImageJ software and are shown (right panel). Thirty conjugates were counted in each of two experiments. Original magnification ×63. ***p < 0.001.

immunological synapse. Because VAV1 phosphorylation was intact in the tolerant T cells, but the enhancement of F-actin at the synapse did not occur, we investigated whether instead VAV1 concentration into the synapse was what was impaired in these cells. Although the background level of staining with anti-VAV1 in P13.9 cells was somewhat high, a careful examination of the interface showed that the tolerant T cells were indeed defective in VAV1 accumulation in the synapse (Fig. 7C). This correlated well with the lack of enhanced actin polymerization (Fig. 7A). Thus, in the adaptively tolerant T cells, improper VAV1 localization seems to contribute to the impaired immunological synapse formation despite VAV1’s intact phosphorylation.

**ITK and GADS are downregulated in adaptively tolerant T cells**

ITK has been reported to be critical for recruiting VAV into the immunological synapse (53). ITK-deficient mice show impaired immune synapse formation, although VAV1 activation is not impaired. Interestingly, ITK protein levels in adaptively tolerant T cells were downregulated ~3-fold (Fig. 8A). Real-time quantitative PCR analysis showed that these T cells also had significantly lower levels (3-fold) of mRNA encoding ITK (Fig. 8B). We also examined the other TEC family kinases (RLK and TEC) by real-time quantitative PCR analysis. The levels of mRNA encoding RLK and TEC were substantially downregulated in both adaptively tolerant T cells and preactivated T cells (Fig. 8B). Thus, ITK is the only family member that is uniquely downregulated in adaptively tolerant T cells.

ITK is normally recruited to the TCR signaling complex by SLP76 after its phosphorylation by activated ZAP70. SLP76, in turn, is brought to the complex by GADS, which recognizes ZAP70-phosphorylated tyrosines on LAT. In adaptively tolerant T cells, we found that SLP76 levels were, if anything, slightly increased, but that GADS levels had decreased ~2- to 3-fold (Fig. 8C). The decrease in GADS was also observed at the mRNA level (Fig. 8B). This combined with the inhibition of LAT phosphorylation greatly reduced the phosphorylation of SLP76 in adaptively tolerant T cells, presumably by preventing SLP76 recruitment to the TCR signaling complex (Fig. 8C). Thus, defective ITK and GADS expression, in addition to the impaired LAT phosphorylation by ZAP70, appear to be the major impediments to VAV1 recruitment to the T cell/APC interface in adaptively tolerant T cells. These defects would also contribute to the impaired phosphorylation of PLCγ1 we noted previously in adaptively tolerant T cells (6).

**Discussion**

The development of an immune synapse between T cells and APCs is a key step in the events leading to full T cell activation. When T cells interact with APCs, their F-actin and signaling molecules are enriched in the specialized junction between the T lymphocyte and the APC, which consists of a central cluster of TCRs surrounded by a ring of adhesion molecules. Actin polymerization at the immune synapse stabilizes conjugate formation and facilitates T cell activation (54). Ise et al. (55) have shown that orally tolerant T cells can form conjugates with APCs, but that they are defective in immunological synapse formation. Their results are consistent with ours in that orally tolerant T cells could not translocate TCR, PKCθ, or lipid rafts to the T cell/APC contact site. Heissmeyer et al. (34) also demonstrated that immunological synapse
formation by in vitro-anergized T cells induced with ionomycin treatment was unstable. Their observations showed by live cell imaging that the synapses formed normally at early time points after incubation on lipid bilayers, but that at later time points, these broke down. Such an analysis remains to be carried out in our adaptive tolerance model.

Integrin-mediated adhesion is essential for the formation of stable contacts between T cells and APCs. RAP1 in T cells is a critical activator of these integrins and plays an essential role in LFA-1–mediated interaction with ICAMs on the APCs (17). Adaptively tolerant T cells showed only modest activation of RAP1 on TCR stimulation, although the basal activity of RAP1 was increased. The almost normal conjugate formation that we observed may be because of the combination of this modest activation of RAP1 and the increased expression of LFA-1 (3-fold) observed on the adaptively tolerant T cells. In other models, VAV1 has been shown to be required for integrin-mediated adhesion of T cells to peptide-loaded APCs (56). The guanine nucleotide exchange factor activity of VAV1 was required for conjugate formation and, to a lesser extent, for integrin activation (57). However, whether VAV recruitment into the synapse is required for the integrin-mediated adhesion of the T cells to peptide-loaded APCs was unclear in these studies. Our data would suggest that VAV activation without enrichment into the central SMAC is sufficient for conjugate formation.

Nonetheless, VAV normally does play an important role in immune synapse formation, TCR capping, and lipid raft clustering into the immune synapse (56, 58). Interestingly, tyrosine phosphorylation of VAV was not impaired in adaptively tolerant T cells and, in fact, was sometimes enhanced after stimulation with Ag-APC or anti-TCR/CD28. In addition, this led to normal activation of RAC1 and CDC42. Because Cbl-b–deficient T cells also show enhanced tyrosine phosphorylation of VAV (59), Cbl-b levels were investigated in adaptively tolerant T cells. However, similar to other models of anergy (60), cbl-b was found to be upregulated instead of downregulated (S. Choi and R. H. Schwartz, unpublished observations). VAV1 can be tyrosine phosphorylated by Lck in vitro (44). Fyn also plays a major role in controlling VAV1 phosphorylation after stimulation through the TCR and CD28 (45). Our studies show that VAV phosphorylation requires Src-family kinase activity in both normal and adaptively tolerant T cells as we could completely inhibit the tyrosine phosphorylation with the SRC-family kinase inhibitor PP2 (Supplemental Figs. 3A, 4A, 4B). We have shown previously that LCK activity is only marginally decreased in adaptively tolerant T cells, and that FYN activity is dramatically increased (6). This enhanced SRC-family
kinase activity might be responsible for the increase in VAV phosphorylation that we sometimes observe. In other cells, this phosphorylation event has been reported to require PI3K-generated lipid products (44) to recruit VAV to the plasma membrane via its pleckstrin homology domain. Furthermore, VAV tyrosine phosphorylation on costimulation of the T cell with anti-CD3/CD28 has been shown to be PI3K dependent (61). In our hands, however, pretreatment with PI3K inhibitors did not reduce VAV tyrosine phosphorylation after stimulation of naive transgenic CD4+ T cells with either anti-TCR/CD4 or anti-CD3/CD28 (Supplemental Fig. 4A) or with MCC peptide-prepulsed APC (Supplemental Fig. 3A), even though the downstream effector of PI3K, AKT, and the downstream effector of AKT, GSK3β, were both normally phosphorylated on anti-CD3/CD28 stimulation, in adaptively tolerant T cells (Supplemental Fig. 4C). Thus, we think that the major pathway for VAV tyrosine phosphorylation is through SRC-family kinases, and that VAV must get to the plasma membrane by some other mechanism.

Despite an intact activation pathway for VAV, RACl, and CDC42 in adaptively tolerant T cells, it was not sufficient to allow VAV to be recruited to the immunological synapse (Fig. 7C). What then is missing? Surprisingly, adaptively tolerant T cells showed decreased expression of ITK at both the mRNA and protein levels. ITK plays an important role in actin polymerization and in VAV recruitment to the immune synapse (62). It also plays a key role in regulating TCR-mediated polarization of integrins and signaling molecules to the site of TCR stimulation, as well as the upregulation of integrin adhesion (63). Like our results, T cells from ITK−/− mice show normal tyrosine phosphorylation of VAV (62, 64). Similarly, loss of ITK expression by siRNA knockdown did not alter the pattern of VAV tyrosine phosphorylation, but instead disrupted the interaction of VAV with SLP76 (53). SLP76 is present in normal amounts in adaptively tolerant T cells; however, interestingly, GADS levels were significantly diminished along with ITK. GADS is a linker protein required to bring SLP76 to the TCR activation complex in the plasma membrane by binding to tyrosine phosphorylation sites on LAT via an SH2 domain interaction (65). LAT phosphorylation, however, is greatly impaired in adaptively tolerant T cells (Fig. 5A), reducing the number of active binding sites for the limited amount of GADS in the cell to find. This combination of negative effects leads to an impairment in SLP76 recruitment to the TCR activation complex. As a consequence, SLP76 cannot be phosphorylated by ZAP70, and this impairs its ability to recruit the limited amount of ITK in the cell to the TCR activation complex. Without ITK mobilization, PLCγ1 is not optimally phosphorylated, leading to an impairment in phosphatidylinositol 4,5-bisphosphate hydrolysis and a failure to generate adequate amounts of the second messengers inositol 1,4,5-triphosphate and diacylglycerol (DAG) (66). In addition, the curtailment of ITK binding limits the recruitment of VAV into the immunological synapse.

As a consequence of these signaling defects, we found that enhancement of actin polymerization at and MTOC polarization toward the T cell/APC contact area were greatly impaired in adaptively tolerant T cells. The actin cytoskeleton is critical for T cell signaling and normal T cell/APC conjugate formation (67). VAV1 regulates this actin polymerization (68); thus, its reduced presence in the synapse would impair F-actin accumulation in this part of the cell. MTOC polarization is controlled by ZAP70 kinase activity (52). Recently, Quann et al. (69) have shown that this polarization is driven by localized accumulation of DAG. Our previous experiments (6) showed impairment in ZAP70 kinase activity.
activity and a profound defect in PLCγ1 phosphorylation in adaptively tolerant T cells. This PLCγ1 defect may result in a limited production of DAG, which could cause the impairment of MTOC polarization seen in these cells.

In this article, we have shown that adaptively tolerant T cells retain the ability to form conjugates with APC but are defective in translocation of TCR signaling molecules into the contact site. The tolerant T cells showed both increased LFA-1 expression levels and increased basal RAP1 activity compared with naïve T cells, even though they could only modestly activate RAP1 on TCR stimulation. These alterations were adequate for initial conjugate formation. In addition, Ag-induced activation of VAV1 was not impaired, only its accumulation into the immunological synapse. IKT has been previously reported to facilitate this recruitment process, and interestingly, IKT levels were downregulated in adaptively tolerant T cells. Also, IKT is normally recruited to the TCR signaling complex by SLP76 after its phosphorylation by activated ZAP70. SLP76 is brought to the complex by GADS, which recognizes ZAP70-phosphorylated tyrosines on LAT. In activated ZAP70, SLP76 is brought to the complex by GADS, and Vα Vβ integrin function by T cell activation: points of convergence and divergence. 

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Disclosures

The authors have no financial conflicts of interest.

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