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Ji-Ji Xie, Jia-Qi Liang, Liang-Hui Diao, Amnon Altman and Yingqiu Li

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TNFR-Associated Factor 6 Regulates TCR Signaling via Interaction with and Modification of LAT Adapter

Ji-Ji Xie,*† Jia-Qi Liang,*† Liang-Hui Diao,* Amnon Altman,† and Yingqiu Li*

TNFR-associated factor (TRAF)6 is an essential ubiquitin E3 ligase in immune responses, but its function in adaptive immunity is not well understood. In this study, we show that TRAF6 is recruited to the peripheral ring of the T cell immunological synapse in Jurkat T cells or human primary CD4+ T cells conjugated with staphylococcal enterotoxin E–pulsed B cells. This recruitment depends on TRAF6 interacting with linker for activation of T cells (LAT) via its TRAF domain. Although LAT was indispensable for TCR/CD28-induced TRAF6 ubiquitination and its ligase activity, RNA interference–induced TRAF6 knockdown in T cells decreased TCR/CD28-induced LAT ubiquitination, tyrosine phosphorylation, and association with tyrosine kinase ZAP70. Overexpression of TRAF6 or its catalytically inactive form C70A promoted and decreased, respectively, LAT tyrosine phosphorylation upon stimulation. Moreover, LAT was ubiquitinated at Lys63 by TRAF6 via K63-linked chain. In addition, TRAF6 was required for and synergized with LAT to promote the TCR/CD28-induced activation of NFAT. These results reveal a novel function and mechanism of TRAF6 action in the TCR–LAT signaling pathway distinct from its role in TCR-induced NF-κB activation, indicating that LAT also plays an adapter role in TCR/CD28-induced activation of TRAF6. The Journal of Immunology, 2013, 190: 4027–4036.

Tumor necrosis factor receptor–associated factor (TRAF)6 belongs to the TRAF family of adapter proteins. It can act as an ubiquitin E3 ligase by inducing K63-linked ubiquitination of target proteins. Unlike other TRAFs, TRAF6 plays a dominant role in NF-κB activation initiated by members of the TNFR superfamily, as well as by members of the IL-1R/TLR superfamily (1–4). In these signaling pathways, receptor engagement results in recruitment of TRAF6 by adapters such as TRIF and MyD88, leading to oligomerization and ubiquitination of TRAF6. TRAF6 then ubiquitinates and activates the TAK1/TAB complex, followed by phosphorylation and activation of the IκB kinase, leading to NF-κB activation (5).

TCR signaling is initiated when the TCR and costimulatory receptors, primarily CD28, on the T cell surface are engaged by cognate Ag presented by APCs. An early TCR signaling event is the activation of the lymphocyte specific protein tyrosine kinase, which then phosphorylates the ITAMs of CD3 complex subunits, thereby facilitating the recruitment and activation of ZAP70. Recruitment of ZAP70 leads to a cascade of phosphorylation events involving linker for activation of T cells (LAT), SH2 domain-containing leukocyte protein of 76 kDa (SLP76), Vav, protein kinase C-δ (PKCδ), and other signaling molecules and eventually activates a number of transcription factors, notably NFAT, NF-κB, and AP-1 (6–11). A polarized dynamic molecular structure called the immunological synapse (IS) or the supramolecular activation cluster (SMAC) is formed at T cell–APC conjugation sites. The mature IS segregates into TCR and PKCδ-rich central SMAC (cSMAC) and an integrin-rich peripheral SMAC (pSMAC) (12). The activation of TCR-proximal molecules and the dynamic IS formation are tightly interwoven temporally and spatially to initiate, balance, amplify, and, eventually, terminate TCR signaling in mature T cells (13). As a result of significant advances in microscopy, smaller aggregates of receptors and signaling molecules, termed microclusters, were found to exist within the IS (13, 14). TCR stimulation leads to the formation of separate IS microclusters containing proteins, such as ZAP70, LAT, and SLP76, which can then fuse or segregate to promote or terminate interactions between signaling proteins, respectively (15, 16).

LAT is a prominent integral membrane adapter protein that plays critical roles in T cell activation (17). The LAT cytoplasmic domain contains several conserved tyrosine residues, including Tyr132, Tyr171, Tyr191, and Tyr226, which are primarily phosphorylated by ZAP70 upon TCR stimulation. These phosphorylated tyrosine residues provide docking sites for the recruitment of adapters (e.g., Grb2, SLP76), enzymes (e.g., PLC-γ1, Vav), and the regulatory subunit of PI3K, resulting in the assembly of a multiprotein complex, the so-called “LAT signalosome.” This signalosome transduces and propagates TCR signals, leading to activation of downstream kinases and transcription factors, including NFAT, and resulting in T cell proliferation and cytokine expression (17–20). Genetic studies and evidence obtained from the analysis of LAT-deficient T cell lines demonstrated that LAT is a vital component of T cell activation, as well as T cell develop-
opment and differentiation, and that it can deliver both positive and negative regulatory signals via its distinct phosphorylated tyrosine residues (21–23). More recently, studies showed that LAT is also subject to ubiquitination, which might be involved in activation-induced internalization of LAT complexes and regulation of LAT protein stability (24–26). Therefore, elucidation of the components and functional mechanisms of the LAT signalingosome is critical to our understanding of T cell activation and immune responses.

TRAF6-mediated ubiquitination and activation of the I KK/NEMO complex plays an essential role in the IL-1R/TLR-induced NF-κB pathway, it is also important for T cell activation, including the TCR-induced NF-κB pathway (27–29). Indeed, TRAF6-deficient mice were shown to display severely abnormal T cell homeostasis (28), a dominant Th2-type polarized autoimmune response (30), resistance to anergizing signals (31), increased Th1 differentiation (32), and enhanced or decreased IL-2 production (28, 31, 33). Hyperactivation of the PI3K-Akt pathway, which is unrelated to NF-κB (34), was suggested to be responsible for the abnormal T cell homeostasis and Th2 polarization in Traf6/−/− mice (28). Although TRAF6 plays important roles in T cell immunity, it remains largely unknown how TRAF6 is regulated and how it functions in the TCR-signaling pathway.

In this study, we found that LAT and TRAF6 regulate each other reciprocally. LAT is required for TRAF6 IS recruitment, ubiquitination, and its E3 activity, whereas TRAF6 associates with LAT through its TRAF domain and functions as an E3 ligase for LAT upon stimulation by promoting K63-linked ubiquitination at Lys48 and, in turn, tyrosine phosphorylation of LAT. Knockdown of TRAF6 inhibited the TCR-induced phosphorylation of PLCγ1 and activation of NFAT, and TRAF6 synergized with LAT to promote TCR-induced NFAT activation. These results suggest that TRAF6 is a component of the LAT signalingosome that functions cooperatively with LAT during the early steps of T cell activation.

**Methods and Materials**

**Plasmids and reagents**

TRAF6 (NCBI Reference Sequence: NP_004611.1) and LAT (NCBI Reference Sequence: NP_001014989.2) cDNAs were amplified by PCR from a cDNA library of Jurkat E6.1 cells and cloned into pFlag-CMV2 (Sigma) and pcDNA3.1-HA (Invitrogen) vectors, respectively. Full-length TRAF6; TRAF6 mutants with deletion of the Ring-Zinc finger (ΔRZF), coil-coil (ΔC), or TRAF domain (ΔTRAF); and TRAF domain–only (TRAF6−TRAF) cDNAs were then subcloned into the pEGFP-C1 (Clontech) and pFlag-CMV2 vectors. Point mutations were introduced by use of a site-directed mutagenesis kit (Stratagene). To construct short hairpin RNA (shRNA) vectors, 60-bp hairpin oligonucleotides were designed and subcloned into pSuper-GFP vectors (Oligogene). TRAF6 was targeted by the following 19-mer sequence: 5′-CCACGAGAGATAGATTCCG-3′ and pSilencer Negative Control: 5′-CCATCCTGATGTCGCAATG-3′. All constructs were confirmed by DNA sequencing. The rabbit Abs to K63-linked ubiquitin (D7A11), LAT phosphorylated at Tyr191, p-ERK (Thr 202/Tyr 204) structures were confirmed by DNA sequencing. The rabbit Abs to K63-linked ubiquitin (AF18), rabbit TRAF6 (H-274), LA T (FL-233), ERK (C-16), Akt (E-19), and HRP-conjugated secondary Abs were purchased from Transduction Laboratories. The mouse ubiquitin (P4D1), SLP76 (F-7), secondary Abs were from Molecular Probes; poly- L-lysine was from Sigma.

From BD Pharmingen, mouse anti-TCR (C305, IgM) was from Upstate, and from Zyme. For stimulation Abs, mouse anti-CD3 Abs (UCHT1, IgG) were from BioLegend, mouse anti-CD28 (290, IgG) and CD28 (2 μg/ml) mAbs on ice, and stimulated at 37°C for the indicated times with gentle shaking by cross-linking with a secondary goat anti-mouse IgG (10 μg/ml; Pierce) or stimulated solely by anti-human CD3 (0.25 μg/ml; IgM). After washing in ice-cold PBS, cells were lysed in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA [pH 8.0], 5 mM Na3P04, 1 mM sodium orthovanadate [Na3VO4], 1 mM PMSF, 1% Nonidet P-40, and 10 μg/ml each aprotinin and leupeptin). For ubiquitination assays, 0.1% SDS was added to the lysis buffer. Lysates were incubated with the indicated Abs plus 30 μl protein G PLUS–Agarose (GE Healthcare) overnight at 4°C with gentle shaking. Samples were washed three times with lysis buffer, and the immunoprecipitates (IPs) were dissolved in 2x Laemmli buffer, subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes, and immunoblotted with the indicated Abs. Densitometry of bands was quantitated by ImageJ software.

**Activation, immunoprecipitation, and immunoblotting**

Cells were washed with serum-free RPMI 1640 medium, serum starved, incubated with anti-human CD3 (10 μg/ml; IgG) and CD28 (2 μg/ml) mAbs on ice, and stimulated at 37°C for the indicated times with gentle shaking by cross-linking with a secondary goat anti-mouse IgG (10 μg/ml; Pierce) or stimulated solely by anti-human CD3 (0.25 μg/ml; IgM). After washing in ice-cold PBS, cells were lysed in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA [pH 7.5], 150 mM NaCl, 5 mM EDTA [pH 8.5], 5 mM Na3P04, 1 mM sodium orthovanadate [Na3VO4], 1 mM PMSF, 1% Nonidet P-40, and 10 μg/ml each aprotinin and leupeptin). For ubiquitination assays, 0.1% SDS was added to the lysis buffer. Lysates were incubated with the indicated Abs plus 30 μl protein G PLUS–Agarose (GE Healthcare) overnight at 4°C with gentle shaking. Samples were washed three times with lysis buffer, and the immunoprecipitates (IPs) were dissolved in 2x Laemmli buffer, subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes, and immunoblotted with the indicated Abs. Densitometry of bands was quantitated by ImageJ software.

**Subcellular fractionation**

Subcellular fractionation of Jurkat T cells was performed as previously described (38). Briefly, the cells were washed in ice-cold PBS, resuspended in ice-cold hypotonic buffer (42 mM KCl, 10 mM HEPES [pH 7.4], 5 mM MgCl2 protease inhibitors), and incubated on ice for 15 min. The cells were transferred to a 1-ml syringe and sheared by passing them five times through a 27-gauge needle. The lysates were centrifuged at 200 × g for 10 min to remove nuclei and cell debris. The supernatant was centrifuged at 25,000 × g for 90 min at 4°C. The supernatant (cytosol) was then col-
Luciferase reporter assays

For reporter assays, Jcam2.5 cells and shTRAF6 (or negative control) Jurkat cells were transfected in triplicates by electroporation with a combination of pNFAT luciferase reporter plasmid and the indicated plasmids. After 24 h, cells were either left unstimulated or were stimulated with cross-linked anti-CD3 plus anti-CD28 mAbs (1 μg/ml each) for 6 h, lysed, and collected for luciferase reporter assay on Berthold Lumat LB 9507. Luciferase activity of cell lysates was measured with a Promega luciferase assay kit, according to the manufacturer’s instructions. Cotransfected Renilla luciferase reporter plasmid was used as an internal control.

Results

TRAF6 is recruited to the pSMAC of the IS

To investigate TRAF6 involvement in T cell activation, we first used confocal microscopy to study its intracellular localization during the formation of conjugates between Jurkat E6.1 T cells and Raji B cells pulsed with a superantigen, SEE, a cellular model used extensively to image the IS in T cells. In parallel, we imaged the localization of PKCθ as a marker of the cSMAC. As shown in Fig. 1A, both TRAF6 and PKCθ were distributed homogeneously throughout the cytoplasm in the resting T cell. Following SEE stimulation, TRAF6 and PKCθ were recruited to the IS within 5 min and were still present there after 30 min. Although PKCθ was localized primarily in the cSMAC, the localization of TRAF6 was more peripheral. Three-dimensional reconstructions of serial z-sections also showed that PKCθ localized mainly in the central synapse, whereas TRAF6 was mainly found in the periphery, consistent with a pSMAC localization (Fig. 1A). Similar results were obtained by conjugating human primary CD4+ T cells and SEE-loaded Raji cells (Fig. 1B).

LAT is required for TRAF6 membrane translocation and IS recruitment

To determine which signaling molecule is required for recruitment of TRAF6 to the IS, we used Jurkat T cell lines deficient in ZAP70 (P116), SLP76 (J14), or LAT (Jcam2.5) and assessed TRAF6 recruitment to the IS following APC/SEE stimulation. TRAF6 accumulation in the IS could be detected in most synapses formed by P116 or J14 cells. However, TRAF6 IS translocation was essentially abolished in Jcam2.5 cells (Fig. 1C). Quantitative analysis of the ratio of TRAF6 immunofluorescence intensity at the T–B cell contact site to the rest of T cell cytosol in 15 conjugates from each cell line showed that, 5 min after T–B cell conjugation, the ratios were 2.23, 2.04, and 1.81 in Jurkat E6.1, P116, or J14 cells, respectively, but only 1.15 in LAT-deficient Jcam2.5 cells (Fig. 1D). This result indicates that LAT, but not ZAP70 or SLP76, is indispensable for TRAF6 recruitment to the IS. Thus, we compared the localization of TRAF6 in Jurkat E6.1 and Jcam2.5 cells by subcellular fractionation. In the absence of stimulation, we observed a constitutive membrane localization of TRAF6, which was increased upon CD3/CD28 costimulation; in contrast, both constitutive and induced membrane translocation of TRAF6 were remarkably diminished in Jcam2.5 cells (Fig. 1E). A more detailed analysis of the spatial relationship between TRAF6 and LAT also revealed that, in resting Jurkat T cells, LAT was detected in the plasma membrane, as well as in intracellular compartments, whereas TRAF6 was distributed homogeneously throughout the cytoplasm with some colocalization with LAT in membrane (Fig. 1F, top panels, arrowheads). After superantigen stimulation, both LAT and TRAF6 translocated to the IS. Z-axis images showed a substantial fraction of LAT, which colocalized with TRAF6 at the IS (Fig. 1F, bottom panels). We also observed membrane coclusters of LAT and TRAF6 outside the IS (Fig. 1F, bottom panels, arrowheads), as well as beneath the IS (Fig. 1F, bottom panels, arrows). These data indicate that LAT is essential for TRAF6 membrane and IS recruitment.

TRAF domain is responsible for TRAF6 IS recruitment and association with LAT

TRAF6 harbors an N-terminal Ring-Zinc finger domain that confers E3 ubiquitin ligase activity, a coil-coil domain important for polymerization, and a conserved C-terminal TRAF domain required for self-association and interaction with receptors (39). To investigate which domain of TRAF6 is important for its IS recruitment, GFP-tagged full-length TRAF6 and several TRAF6 mutants were generated: a ∆RZF (Δ1–288) mutant lacking the first 288 aa that contains the Ring-Zinc finger; a ACC (∆289–350) mutant lacking the coil-coil domain; a ∆TRAF (∆351–522) mutant lacking the C-terminal TRAF domain; and TRAF domain-only (351–522) protein (Fig. 2A). We then analyzed the localization of the GFP-TRAF6 fusion proteins in Jurkat T cells engaged by Raji B cells pulsed or not with SEE superantigen. PKCθ localization was also examined as an IS marker.

In T cells conjugated with control (non–SEE-pulsed) Raji cells, all of the GFP-TRAF6 proteins, as well as PKCθ, were distributed homogeneously in the submembrane cytoplasmic region surrounding the large nucleus. After conjugation with SEE-pulsed Raji cells, the majority of the GFP-TRAF6, GFP-TRAF6-∆RZF, and GFP-TRAF6-ACC proteins were found to accumulate diffusely all over the T–B cell contact area. Notably, GFP-TRAF6-TRAF displayed a characteristic pSMAC distribution. In contrast, there was no accumulation of GFP-TRAF6-∆TRAF in the IS (Fig. 2B). Interestingly, the TRAF6 catalytically inactive point mutant C70A displayed normal IS translocation, suggesting that TRAF6 relocalization is independent of its E3 ligase activity (Fig. 2B). The IS recruitment of wild-type TRAF6 and its mutants in Fig. 2B was also analyzed quantitatively (Fig. 2C). These data indicate that the TRAF domain of TRAF6 is necessary and sufficient for its targeting to the IS.

Next, we explored the role of the TRAF domain in the interaction between TRAF6 and LAT. HEK293T cells were cotransfected with LAT together with an empty vector, wild-type TRAF6, or each of several TRAF6 mutants. We then performed reciprocal immunoprecipitations of transfected TRAF6 or LAT by using the relevant tag-specific Abs. Although GFP-LAT coimmunoprecipitated with wild-type TRAF6 and its catalytically inactive mutant C70A, deletion of the TRAF domain abolished the association with GFP-LAT (Fig. 2D). Similar results were observed when anti-HA IPs of HA-tagged LAT were analyzed for the presence of TRAF6 (Fig. 2E). As expected, the isolated TRAF domain could pull down LAT as well; in fact, it was even more effective than wild-type TRAF6 in that regard (Fig. 2F). Of note, upshifted bands above LAT monomers were also observed when LAT and wild-type TRAF6 were coexpressed (Fig. 2D, 2E, 2F). We also explored structural features of LAT required for TRAF6 binding. LAT contains a short extracellular region, a single transmembrane-spanning region, and a long intracellular region with no apparent intrinsic enzymatic activity or protein–protein interaction domains. However, the LAT cytoplasmic domain contains several conserved tyrosines rapidly phosphorylated upon TCR engagement that provide docking sites for the recruitment of adapters (20). To examine whether the tyrosine phosphorylation–dependent activation of LAT is required for its interaction with TRAF6, we constructed LAT truncations without the four distal tyrosines (1–132) or retaining only the PLC-ζ1-binding site (mediates Nfat activation (1–195)) (NP_001014989.2, longer LAT isoform; Supplemental Fig. 1). Full-length GFP-LAT and the two truncations, but not the GFP vector, coimmunoprecipitated with...
Flag-TRAF6, indicating that the activation of LAT was dispensable for TRAF6 association (Fig. 2G). Together, these results indicate that TRAF6 translocates to the IS via binding LAT through its TRAF domain, which is consistent with the results shown in Fig. 1.

LA T is required for TRAF6 ubiquitination and its E3 ligase activity

Because LAT associates with TRAF6 and is required for its IS recruitment, we analyzed whether LAT influences TRAF6 activity. Because TRAF6 ubiquitination correlates with its activation (40, 41), we compared the ubiquitination status of TRAF6 in wild-type Jurkat E6.1 cells versus LAT-deficient Jcam2.5 cells. We found that, following anti-CD3/CD28 costimulation, the ubiquitination of TRAF6 was increased significantly in Jurkat E6.1 cells but not in Jcam2.5 cells, and even the weak constitutive ubiquitination of TRAF6 was not detectable in Jcam2.5 cells (Fig. 3A). We also determined the TCR/CD28-induced K63 ubiquitination of TAK1.

**FIGURE 1.** LAT is required for TRAF6 pSMAC recruitment. (A) Raji B cells labeled with CellTracker Blue were pulsed or not with SEE superantigen and allowed to interact with Jurkat E6.1 T cells for the indicated lengths of time. Conjugates were fixed and labeled with Abs to TRAF6 and cSMAC marker PKCζ. One representative section of each sample analyzed by confocal microscopy is shown. Overlay of the TRAF6 (red, Alexa Fluor 594) or PKCζ (green, Alexa Fluor 488) and Raji B cells (blue, CellTracker Blue) images are shown. The z-axis stacks (40 images) were taken at 0.2-μm increments. The reconstructed cell is shown in the z-plane. Three-dimensional images were obtained with Imaris software. The result represents four independently performed experiments. Scale bar, 5 μm. (B) Primary CD4+ T cells isolated from human PBMCs were treated as in (A). Overlay of the TRAF6 (red, Alexa Fluor 594) or PKCζ (green, Alexa Fluor 488) and Raji B cells (blue, CellTracker Blue) images are shown. The z-axis stacks (40 images) were taken at 0.2-μm increments. The result represents two independently performed experiments. Scale bar, 5 μm. (C) Wild-type and mutated Jurkat E6.1 cells were incubated with SEE-pulsed or nonpulsed CellTracker Blue–labeled Raji B cells for 5 min and then processed as in (A). Fluorescence intensity curves are shown. Data are representative of 15 conjugates. Red (Alexa Fluor 594), TRAF6; green (Alexa Fluor 488) TCR as an IS marker; blue (CellTracker Blue), Raji B cells. Images are representative of three independent experiments. Scale bar, 5 μm. (D) The ratio of TRAF6 immunofluorescence intensity in the T cell–APC contact site relative to the rest of the T cell cytosol. The horizontal lines represent the mean ± SD value of 15 conjugates from each condition. (E) Cytosolic and membrane fractions were prepared from Jurkat E6.1 and Jcam2.5 cells stimulated with anti-CD3 (10 μg/ml) and anti-CD28 (2 μg/ml) mAbs for different times. Extracts were immunoblotted with the indicated Abs. Expression of calnexin in the membrane fractions and actin in the cytosolic fractions served as loading controls and confirmed proper fractionation. The result represents three independently performed experiments. (F) TRAF6 colocalization with LAT in resting and stimulated T cells, representative of three independent experiments. Jurkat TAG cells were transfected with GFP-TRAF6, incubated with SEE-pulsed or nonpulsed CellTracker Blue–labeled Raji B cells for 5 min, fixed, and labeled with Abs to LAT or PKCζ. One representative section of each sample analyzed by confocal microscopy is shown. Arrows indicate subsynaptic LAT vesicles colocalizing with cytosolic TRAF6, and arrowheads indicate LAT colocalization with membrane TRAF6. The z-axis stacks (40 images) were taken at 0.2-μm increments. Images are representative of three independent experiments. Magenta (Alexa Fluor 647), PKCζ; red (Alexa Fluor 594), LAT; green, GFP-TRAF6. Scale bar, 5 μm.
which is a known substrate of TRAF6 E3 ligase, and found it to be dramatically decreased in Jcam2.5 cells compared with Jurkat E6.1 cells (Fig. 3B). Because Akt is another direct substrate of TRAF6 (42), we further examined the activities of TRAF6 by incubating the TRAF6 immunoprecipitated from stimulated Jurkat T cells with HEK293T cell lysate and then checking the K63 ubiquitination of Akt from HEK293T cell lysates. We found that the TRAF6 immunoprecipitated from Jurkat E6.1 cells could ubiquitinate Akt efficiently, whereas the TRAF6 immunoprecipitated from Jcam2.5 cells could not (Fig. 3C). These results indicate that TRAF6 E3 ubiquitination ligase activity induced by TCR/CD28 stimulation is greatly impaired in Jcam2.5 cells, thus LAT is required for TRAF6 ubiquitination and E3 ligase activity in the TCR-signaling pathway.

TRAF6 promotes LAT ubiquitination and phosphorylation upon TCR engagement

To better understand the function of TRAF6 in T cells, we constructed a stable TRAF6 knockdown (shTRAF6) and a negative control Jurkat E6.1 cell line, using a TRAF6-specific shRNA sequence and a control, scrambled shRNA sequence, respectively. The shTRAF6 cells displayed a markedly decreased level of CD3/CD28 stimulation is greatly impaired in Jcam2.5 cells, thus LAT is required for TRAF6 ubiquitination and E3 ligase activity in the TCR-signaling pathway.
CD28-induced LAT ubiquitination and tyrosine phosphorylation compared with negative control cells, whereas the LAT protein levels were very similar in both cell lines (Fig. 4A, 4B). Consistent with data of King et al. (28), phosphorylation of Akt was enhanced with data of King et al. (28), phosphorylation of Akt was enhanced upon anti-CD3 (10 μg/ml) and anti-CD28 (2 μg/ml) mAbs for the indicated times, lysed with lysis buffer containing 0.1% SDS to eliminate noncovalent interactions, immunoprecipitated with rabbit anti-TRAF6 or goat anti-mouse IgG, and probed with the indicated Abs. The results are representative of four independent experiments. (B) Jurkat E6.1 cells and Jcam2.5 cells were left unstimulated or were stimulated with anti-CD3 (10 μg/ml) or anti-CD28 (2 μg/ml) mAbs. The results are representative of two independent experiments. (C) Jurkat E6.1 cells and Jcam2.5 cells were left unstimulated or were stimulated with anti-CD3 (10 μg/ml) or anti-CD28 (2 μg/ml) mAbs. Results are representative of two independent experiments.

**FIGURE 3.** LAT is required for TCR-induced TRAF6 ubiquitination and its E3 ligase activity. (A) Jurkat E6.1 cells and Jcam2.5 cells were left unstimulated or were stimulated with anti-CD3 (10 μg/ml) and anti-CD28 (2 μg/ml) mAbs for the indicated times, lysed with lysis buffer containing 0.1% SDS to eliminate noncovalent interactions, immunoprecipitated with rabbit anti-TRAF6 or goat anti-mouse IgG, and probed with the indicated Abs. The results are representative of four independent experiments. (B) Jurkat E6.1 cells and Jcam2.5 cells were left unstimulated or were stimulated as in (A) for the indicated times, lysed with lysis buffer containing 0.1% SDS, immunoprecipitated with rabbit anti-TAK1 or goat anti-mouse IgG, and probed with the indicated Abs. Results are representative of two independent experiments. (C) Jurkat E6.1 cells and Jcam2.5 cells were stimulated for 20 min as in (A), lysed with lysis buffer containing 0.1% SDS, immunoprecipitated with rabbit anti-TRAF6 or goat anti-mouse IgG, and incubated with a lysate of HEK293T cells for 1 h at 30°C. The incubations were stopped by centrifuge, the pellets were saved, and the supernatants were subjected to further immunoprecipitation with anti-Akt Ab and probed with the indicated Abs. Results are representative of two independent experiments.

LAT and ZAP70 and, thus, promotes tyrosine phosphorylation of LAT. TRAF6 cooperates with LAT in CD3/CD28-induced activation of NFAT

Decreased LAT Y132 phosphorylation was observed in activated T cells after knockdown of TRAF6 (Fig. 4A). LAT Y132 was shown to be critical for binding and phosphorylation of the signaling mediator PLC-γ1, which, in turn, activates the downstream NFAT, an important target of LAT in T cell activation (17). Thus, we examined whether TRAF6 could influence PLC-γ1 activation and membrane recruitment and then the activation of NFAT. PLC-γ1 phosphorylation was reduced in shTRAF6 cells after CD3/CD28 stimulation, whereas the PLC-γ1 protein levels were similar (Fig. 5A). Subcellular fractionation results revealed that TRAF6 knockdown did not affect stimulation-induced PLC-γ1 membrane translocation (Fig. 5B), consistent with the notion that membrane recruitment of PLC-γ1 does not necessarily result in phosphorylation in Jurkat T cells; in contrast, overexpression of TRAF6-C70A decreased LAT Tyr191 phosphorylation (Fig. 4E, 4F). As the tyrosine kinase for LAT, ZAP70 microclusters contact with LAT and SLP76 microclusters dynamically (15–17). Additional experiments revealed that the transient association of LAT with ZAP70 or SLP76, which occurred instantly after TCR ligation, was not present in stimulated shTRAF6 cells (Fig. 4G). To avoid the artificial immunoprecipitation by the stimulatory Ab, an IgM anti-TCR Ab was used in the absence of anti-CD28 or anti-IgG (Fig. 4G). These data suggest that the TCR-induced and TRAF6-dependent ubiquitination of LAT facilitates the association between LAT and ZAP70 and, thus, promotes tyrosine phosphorylation of LAT.

**FIGURE 4.** TRAF6 is an E3 ubiquitin ligase for LAT

To investigate whether TRAF6 is an E3 ligase for LAT, Jurkat T cells were transfected with wild-type TRAF6 or its E3 ligase–inactive mutant C70A, and cell lysates were immunoblotted with a K63 ubiquitin-specific Ab. We found that LAT K63-linked ubiquitination was enhanced in TRAF6-expressing cells but not in TRAF6 C70A-expressing cells (Fig. 5A), suggesting that TRAF6 is an E3 ubiquitin ligase for LAT. This notion is consistent with the observation of higher molecular weight forms of LAT when coexpressed with wild-type TRAF6 in HEK293T cells (Fig. 2D, 2F, C). Inspection of the LAT amino acid sequence revealed only two lysines (K88 and K240 in human LAT, NP_001014989.2, longer LAT isoform) (Supplemental Fig. 1). To validate that TRAF6 may ubiquitinate LAT at K88. In anti-CD3/CD28-stimulated Jurkat T cells, K63-linked ubiquitination and Tyr191 phosphorylation were observed in IPs of wild-type LAT and the LAT K240R mutant, but not in the LAT K88R IPs, implying that TCR induces ubiquitination of LAT at K88 (Fig. 5C). To validate that TRAF6-derived LAT ubiquitination at K88 is TCR induced, we compared the K63-linked ubiquitination status of HA-LAT or its K240R mutant before and after knockdown of TRAF6 protein upon TCR stimulation. We found that knockdown of TRAF6 greatly blocked the TCR-induced K63-ubiquitin conjugation at LAT K240R, whereas the K63-ubiquitin conjugation of LAT K240R was almost the same as that of wild-type LAT in control cells (Fig. 5D). These data indicate that TCR induces the K63 ubiquitination of LAT on K88 by TRAF6.

**FIGURE 5.** LAT and TRAF6 cooperate in CD28-induced and CD3-induced signaling of T cells

Increased LAT Y132 phosphorylation was observed in activated T cells after knockdown of TRAF6 (Fig. 4A). LAT Y132 was shown to be critical for binding and phosphorylation of the signaling mediator PLC-γ1, which, in turn, activates the downstream NFAT, an important target of LAT in T cell activation (17). Thus, we examined whether TRAF6 could influence PLC-γ1 activation and membrane recruitment and then the activation of NFAT. PLC-γ1 phosphorylation was reduced in shTRAF6 cells after CD3/CD28 stimulation, whereas the PLC-γ1 protein levels were similar (Fig. 6A). Subcellular fractionation results revealed that TRAF6 knockdown did not affect stimulation-induced PLC-γ1 membrane translocation (Fig. 6B), consistent with the notion that membrane recruitment of PLC-γ1 does not necessarily result in...
PLC-γ1 activation (43). Next, negative control and shTRAF6 Jurkat T cells were transfected with NFAT-luciferase reporter gene, and luciferase activity was determined after costimulation with anti-CD3/CD28 mAbs for 6 h. Although control cells displayed an ~3-fold increase in NFAT activity after stimulation, shTRAF6 cells showed a minimal increase (~1.5-fold) (Fig. 6C).
TRAF6 AND LAT REGULATE EACH OTHER RECIPROCALLY IN T CELLS

Discussion

TRAF6 is a key ubiquitin E3 ligase in NF-κB–mediated immune responses. However, its involvement in TCR signaling remains largely undefined. In this study, we show that TRAF6 was recruited to the pSMAC of the T cell IS and that this localization depended on its association with LAT via the TRAF domain. LAT was required for the TCR-induced TRAF6 ubiquitination and its K63-linked ubiquitin E3 ligase activity. Conversely, TRAF6 was important for the TCR-induced K63-linked ubiquitination of LAT and for its association with ZAP70, tyrosine phosphorylation, and LAT-mediated NFAT activation. Thus, our results reveal a novel layer of TRAF6 regulatory activity in TCR signaling mediated by its interplay with LAT.

As a master adapter that couples TCR signaling to downstream signaling events, LAT mainly supplies phosphorylated tyrosine residues that function as docking sites for the recruitment and activation of different effectors. In this study, we showed that, in the absence of LAT, the TCR-induced ubiquitination and IS recruitment of TRAF6, as well as its E3 ubiquitin ligase activity, were essentially missing. Thus, LAT is a critical adapter for TCR-induced recruitment and activation of TRAF6. A TRAF6-binding motif, PxxxD/E/F, which binds the TRAF domain of TRAF6, was found in upstream adapter proteins that, together with TRAF6, play important roles in CD40 and IL-1R/TLR signaling (44). MALT1, a crucial molecule in TCR-induced NF-κB activation, serves as an adapter for TRAF6 and associates with it through two TRAF6-binding motifs (27). In the case of LAT-TRAF6 interaction, as in the case of other TRAF6-associated proteins, the TRAF domain is responsible for binding LAT. However, LAT does not have a PxxxD/E/F motif, suggesting that LAT uses either a different motif or an intermediate adapter to bind the TRAF domain of TRAF6. Because gene-knockout studies demonstrated distinct effects of LAT and TRAF6 versus MALT1 on T cell activation (22, 28, 45), MALT1 does not seem to be the adapter that mediates the association between TRAF6 and LAT. Thus, TRAF6 apparently interacts with LAT and MALT1, via different mechanisms within two different complexes.

We identified Lys^{88} as the LAT site that undergoes TRAF6-induced K63-linked ubiquitination. This finding is consistent with a report that LAT is ubiquitinated at this site (26). However, in our study, K63-linked ubiquitination of LAT by TRAF6 was associated with increased LAT phosphorylation, whereas Samelson and colleagues (26) reported that LAT ubiquitination by Cbl proteins is important for LAT protein stability. This apparent difference raises the question of how a single site can be modified by two different E3 ligases that lead to distinct functional outcomes. Because TRAF6 knockdown can affect LAT ubiquitination and phosphorylation at very early stimulation times, it is possible that this conundrum could reflect successive LAT modification, where TRAF6 functions early to modify LAT, whereas Cbl proteins regulate LAT half-life at later activation stages.

Interestingly, this study demonstrated that the membrane-proximal region of LAT preceding Tyr^{132} mediates its association with TRAF6, which promotes the ubiquitination of LAT and, in turn, the phosphorylation of tyrosine residues on LAT. This finding assigns a novel function to this membrane-proximal region, namely, association

indicating a substantial inhibition in NFAT activation as a result of TRAF6 knockdown. Additional experiments were performed in LAT-deficient Jcam2.5 cells, which were transfected with NFAT-luciferase reporter gene plus LAT, together with wild-type TRAF6 or various mutants. NFAT activation was abolished in anti-CD3/CD28–costimulated Jcam2.5 cells, with or without TRAF6 transfection (Fig. 6D). LAT expression alone increased NFAT activity by ~2-fold, whereas coexpression of wild-type TRAF6 and LAT resulted in a dramatic, ~8-fold increase in NFAT activity following stimulation (Fig. 6D). Substantial (~6-fold) NFAT activation was also observed in cells expressing the IS-localized TRAF6-ΔCC mutant. In contrast, coexpression of the TRAF6-ΔTRAF or TRAF6 C70A mutants did not augment the weak level of NFAT activation induced by LAT alone (Fig. 6D). These data indicate that TRAF6 positively regulates LAT-induced NFAT activation in T cells; furthermore, the ligase activity of TRAF6 E3 and its TRAF domain are both needed for the proper function of TRAF6 in the TCR-mediated, LAT-dependent signaling pathway leading to NFAT activation.

FIGURE 5. TRAF6 is an E3 ubiquitin ligase for LAT. (A) Jurkat TAg cells were transfected with the indicated constructs. After 24 h, the cells were lysed with lysis buffer supplemented with 0.1% SDS, immunoprecipitated with rabbit anti-HA, and probed with the indicated Abs. Data are representative of two independent experiments. (B) Jurkat TAg cells were transfected with the indicated constructs. After 24 h, the cells were lysed with lysis buffer, immunoprecipitated, and probed with the indicated Abs as in (A). Data are representative of three independent experiments. (C) Jurkat TAg cells were transfected with the indicated constructs. Twenty-four hours later, the transfected cells were either left untreated or stimulated with anti-CD3 (10 μg/ml) and anti-CD28 (2 μg/ml) Abs for the indicated times, lysed with lysis buffer supplemented with 0.1% SDS, immunoprecipitated with rabbit anti-HA, and probed with the indicated Abs. Results are representative of two independent experiments. (D) Jurkat TAg cells were transfected with the indicated constructs and siRNAs. Forty-eight hours later, the transfected cells were either left untreated or stimulated with anti-CD3 (10 μg/ml) and anti-CD28 (2 μg/ml) Abs for the indicated times, lysed with lysis buffer supplemented with 0.1% SDS, immunoprecipitated with rabbit anti-HA, and probed with the indicated Abs. Results are representative of four independent experiments.

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with TRAF6, which is required for phosphorylation of the more distal tyrosine residues in LAT.

Deletion of Lat in mature CD4+ T cells leads to a lymphoproliferative disorder accompanied by elevated Th2 cytokine production (22, 46). Meanwhile, Traf6 deletion has no apparent effect on TCR-induced NF-κB activation but instead leads to severe T cell hyperproliferation and a Th2-dominant autoimmune response, which may result from hyperactivation of the PI3K-Akt pathway (28, 29). We showed in this study that TRAF6 knockdown significantly decreases LAT tyrosine (including Tyr 132 and Tyr 191) phosphorylation. Phosphorylated Tyr 191 residue in human LAT (Tyr195 in murine LAT) recruits Gab2, which acts as a scaffold protein for the phosphatase SHP-2 and a negative regulator of the PI3K-Akt pathway (28, 29). Therefore, TRAF6-dependent enhancement of LAT Tyr191 phosphorylation may provide a molecular, mechanistic basis for the similarity between the T cell phenotypes of Traf6-deficient mice and Lat-deficient mice, as well as for the T cell–intrinsic negative-regulatory role of TRAF6 and LAT (28, 46, 47). Moreover, impaired LAT Tyr132 phosphorylation in TRAF6-knockdown T cells could be the major reason for decreased phosphorylation of PLC-γ1 and the subsequent inhibition of NFAT activation upon TCR engagement. Thus, this discovery may also reveal an underlying molecular link between the requirement of TRAF6 and the function of NFAT in T cell anergy (31, 48, 49).

Intriguingly, knockdown of TRAF6 did not affect the TCR-induced membrane recruitment of PLC-γ1 (Fig. 6B), despite reducing its TCR-induced tyrosine phosphorylation, as well as that of LAT. This reduced phosphorylation may result from deficient recruitment of ZAP-70 to the LAT signalosome (Fig. 4G). Regarding the unabated membrane recruitment, it is known that membrane recruitment of PLC-γ1 is not solely dependent on its binding to phosphorylated Tyr132 of LAT; other proteins, including SLP76, which forms separate microcluster distinct from LAT, may also contribute to this membrane recruitment (15, 20, 43).

In summary, we discovered that the adapter LAT is essential for TCR-induced TRAF6 E3 ligase activity and that, in turn, TRAF6 regulates LAT tyrosine phosphorylation by promoting K63-linked ubiquitination of LAT. As a result of this reciprocal cross-talk, TRAF6 and LAT cooperate and apparently synergize to enhance TCR-induced NFAT activation. Our results reveal a previously unknown mechanism underlying the immunological function of TRAF6 in T cells and, furthermore, highlight a novel regulatory mechanism of TCR proximal signaling.

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Disclosures
The authors have no financial conflicts of interest.

References