Tripartite motif containing protein 27 negatively regulates CD4 T cells by ubiquitinating and inhibiting the class I PI3K-C2β

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The K+ channel KCa3.1 is required for Ca2+ influx and the subsequent activation of CD4 T cells. The class II phosphatidylinositol 3-kinase C2β (PI3KC2β) is activated by the T-cell receptor (TCR) and is critical for KCa3.1 channel activation. Tripartite motif containing protein 27 (TRIM27) is a member of a large family of proteins that function as Really Interesting New Gene (RING) E3 ubiquitin ligases. We now show that TRIM27 functions as an E3 ligase and mediates lysine 48 polyubiquitination of PI3KC2β, leading to a decrease in PI3K enzyme activity. By inhibiting PI3KC2β, TRIM27 also functions to negatively regulate CD4 T cells by inhibiting KCa3.1 channel activity and TCR-stimulated Ca2+ influx and cytokine production in Jurkat, primary human CD4 T cells, and TH1 and TH2 CD4 T cells generated from TRIM27−/− mice. These findings provide a unique mechanism for regulating class II PI3Ks, and identify TRIM27 as a previously undescribed negative regulator of CD4 T cells.

PI3Ks play critical roles in the regulation of a variety of biological processes. In general, members of this family have been divided into three classes (I, II, III) based on sequence homology and substrate specificity (2–4). Most of the previous work on PI3Ks in lymphocyte activation have focused on the class I PI3Ks (p110α, β, γ, and δ), which are responsible for the acute rise in P(3,4,5)P3 following antigen-receptor activation (5, 6). Studies in knockout mice have demonstrated that p110β and p110δ play partly redundant functions in T-cell activation, and are most important for T-cell receptor (TCR) signaling by peripheral T cells, as well as for T-cell development and survival (7–11). Although the exact role for P(3,4,5)P3 in T-cell activation is still controversial, recruitment and activation of a number of plekstrin-homology-containing proteins by P(3,4,5)P3 is critical. For example, recruitment of the T cell-specific kinase ITK to the plasma membrane via binding of its plekstrin-homology domain to P(3,4,5)P3 has been shown to contribute to Ca2+ influx, integrin activation, and synapse formation (12). Mammals have three class II PI3Ks: PI3KC2α, PI3KC2β, and PI3KC2γ (1, 3). Although PI3KC2α and PI3KC2β have a wide tissue distribution and are both expressed in lymphocytes, PI3KC2γ has a more restricted pattern of expression and is absent from lymphocytes. Unlike the class I PI3Ks, the class II PI3Ks do not contain regulatory subunits (3, 13). Rather, upstream activation of class II PI3Ks is likely mediated by their extended N and C termini. Agonist-induced relocalization of a constitutively active class II PI3K to the plasma membrane via interaction of their N and C termini with adaptor signaling molecules, such as Grb2 in EGFR signaling (14), or with membrane-associated complexes, such as clathrin or intersectin, have been previously described (15, 16). In addition, some studies have shown agonist-induced increase in kinase activity (4, 17, 18).

Recently, we found that the class II PI3KC2γ plays an important and unexpected role in CD4 T-cell activation (19). These studies demonstrated that activation of PI3KC2γ, but not PI3KC2β, following TCR stimulation functions to recruit PI3KC2β to the immunological synapse, leading to the generation of P(3,5)P3, which is subsequently required for the histidine phosphorylation and activation of KCa3.1 by nucleoside diphosphate kinase B (NDPK-B) (19, 20). Activation of KCa3.1, as well as another K+ channel, Kv1.3, has been shown to play critical roles in CD4 T-cell activation (21–25). By mediating the efflux of K+, these channels function to maintain a negative membrane potential, which is critical for sustained calcium entry into these cells via calcium release-activated Ca2+ channels. Increased cytosolic Ca2+ then mediates the transcriptional activation of a number of genes critical for T-cell activation (26–28).

To understand the mechanism whereby PI3KC2β is regulated in T cells, we screened for PI3KC2β interacting proteins by yeast two-hybrid and identified TRIM27 (also known as Ret finger protein) as a PI3KC2β interacting protein. TRIM family proteins are characterized by the presence of the tripartite motif, which consists of a ring finger, Zn2+ binding motifs referred to as “B boxes,” and a coiled-coil domain (29, 30). TRIM family members have been shown to regulate a plethora of cellular pathways, including apoptosis, the cell cycle, and antiviral activity, and recent evidence has indicated that this family of proteins regulates some of these processes by functioning as a novel class of Really Interesting New Gene (RING) E3 ubiquitin ligases (29–31). We now show that by ubiquitinating PI3KC2β, TRIM27 inhibits PI3KC2β’s kinase activity, resulting in decreased KCa3.1 channel activity and decreased TCR-stimulated Ca2+ influx and cytokine production, thereby identifying TRIM27 as a unique negative regulator of CD4 T cells.

Results

TRIM27 Associates with PI3KC2β in the Yeast Two-Hybrid and in Vivo.

Four TRIM27 clones were identified that bound PI3KC2β in a yeast two-hybrid screen of a human CD4 T-cell library (Hybergenics). Although two clones encompassed full-length TRIM27, these clones contained only the carboxyl-terminal PRY-SPRY domain (also known as B30.2), indicating that these domains are sufficient for binding at least in vitro. To determine whether TRIM27 and PI3KC2β associate in cells, GFP-PI3KC2β or GFP-PI3KC2α was cotransfected with FLAG-TRIM27 in HEK293 cells and association was assessed by coimmunoprecipitation experiments. FLAG-TRIM27 coimmunoprecipitated with anti-GFP antibodies (Fig. 1A, lane 5), and GFP-PI3KC2β coimmunoprecipitated with anti-FLAG antibodies (Fig. 1A, lane 5). The association was specific because FLAG-TRIM27 only coimmunoprecipitated with GFP-PI3KC2β when both proteins were expressed (Fig. 1A, lanes 1 and 3). FLAG-TRIM27 also


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coimmunoprecipitated with the closely related class II PI3K, PI3KC2γ (Fig. 1A, lane 4), although the association was decreased compared with PI3KC2β. In addition, endogenous TRIM27 coimmunoprecipitated with endogenous PI3KC2β, providing further validation that the association of the two proteins is physiologic (Fig. 1C).

To identify the subcellular localization of TRIM27 and PI3KC2β, mCherry-PI3KC2β and GFP-TRIM27 were expressed in COS cells and subcellular localization was assessed by fluorescence. When expressed alone, mCherry-PI3KC2β was predominantly cytosolic, although a portion was associated with the plasma membrane (Fig. 1B). In contrast, virtually all of the expressed GFP-TRIM27 was found localized to a poorly described subcellular vesicular compartment (Fig. 1B). Coexpression of mCherry-PI3KC2β together with GFP-TRIM27 resulted in recruitment of a portion of the coexpressed PI3KC2β to the TRIM27 subcellular compartment (Fig. 1B). Thus, these data indicate that TRIM27 and PI3KC2β associate in vivo and TRIM27 functions to localize a portion of PI3KC2β to a poorly described subcellular compartment, at least in COS cells.

To address the subcellular localization of TRIM27, GFP-TRIM27 overexpressing cells were incubated with rhodamine-transferrin to label the transferrin receptor, lysotracker to label lysosomes, or mitotracker to label mitochondria. These studies demonstrated that a portion of TRIM27 partially overlaps with the transferrin receptor and is likely in recycling endosomes, whereas none of the GFP-TRIM27 localized to the lysosome or mitochondria (Fig. S1).

**TRIM27 Ubiquitinates PI3KC2β in Vivo.** TRIM family members are a class of novel ring-finger E3 ligases that have been shown to ubiquitinate a number of different proteins (30–32). To assess whether TRIM27 ubiquitinates PI3KC2β, PI3KC2β was expressed with His-tagged ubiquitin either alone or together with TRIM27 and ubiquitinated PI3KC2β was assessed following purification with Nickel (Ni)-NTA beads. Although ubiquitinated PI3KC2β was detected when coexpressed with His-tagged ubiquitin alone, the amount of ubiquitinated PI3KC2β was further increased when coexpressed with TRIM27 (Fig. 2A and B). Ubiquitination was specific because TRIM27 failed to ubiquitinate PI3KC2γ (Fig. 2B).

In addition, TRIM27-stimulated ubiquitination of PI3KC2β required TRIM27’s RING domain; a TRIM27 RING mutant (RING MT) did not stimulate an increase in PI3KC2β ubiquitination (Fig. 2A) [RING, mutation of the zinc binding domain, as previously described (33)], even though PI3KC2β (RING MT) still bound and colocalized with TRIM27 in vivo. TRIM27 also stimulated the ubiquitination of a kinase dead PI3KC2β (KD, K805R) (Fig. 2A).

To address whether endogenous TRIM27 ubiquitinates PI3KC2β, ubiquitination was assessed as above following transfection with an siRNA to TRIM27. Consistent with endogenous TRIM27 functioning as an E3 ligase for PI3KC2β, PI3KC2β ubiquitination was markedly decreased in cells transfected with two different siRNAs to TRIM27 (Fig. 2C, lanes 3 and 4); this was specific because a control siRNA did not inhibit (Fig. 2C, ctrl). In addition, the decrease in PI3KC2β ubiquitination in TRIM27 siRNA-transfected cells could be rescued by expressing exogenous TRIM27 that did not bind the TRIM27 siRNA (Fig. 2C, lane 5).

**TRIM27 Stimulates K48 Polyubiquitination of PI3KC2β.** Ubiquitination occurs predominantly via K48 or K63 linkages and consists of the addition of a single ubiquitin (monoubiquitination) or the assembly of ubiquitin of various lengths that are assembled by the successive attachment of ubiquitin to lysines of previously conjugated ubiquitin (polyubiquitination) (34). Although ubiquitin contains seven lysine residues that have been shown to mediate ubiquitin conjugation, K48 and K63 linkages are the best studied. To assess whether TRIM27 mediates K48, K63, mono-, or polyubiquitination of PI3KC2β, Western blotting was performed using antibodies that distinguish between these various ubiquitin modifications (35). TRIM27-stimulated ubiquitination of PI3KC2β reacted with the Lys48-specific antibody (Millipore; clone Apu2) and polyubiquitin-specific antibody (Millipore; clone FK1), and did not react with the Lys63-specific antibody (Millipore; clone HWA4C4) (Fig. 3).

**Ubiquitination of PI3KC2β Inhibits PI3KC2β Kinase Activity and Does Not Stimulate Degradation.** K48 polyubiquitination has been shown to mediate proteosomal degradation of modified proteins, although all non-K63-mediated ubiquitination can target proteins for degradation (36, 37). To determine whether TRIM27 stimulated the degradation of PI3KC2β, HEK293 cells were transfected with TRIM27(WT) or TRIM27(RING MT) and the amount of endogenous PI3KC2β was assessed by Western blot with or without treatment with the proteosome inhibitor MG132. These findings demonstrated that the total amount of PI3KC2β was similar between TRIM27 and control transfected cells, and the amount of PI3KC2β was unaffected by treatment with MG132 (Fig. 4A). In addition to our finding that TRIM27 ubiquitinates PI3KC2β, previous studies have demonstrated that TRIM27 also undergoes autoubiquitination (31). To assess whether either TRIM27 or PI3KC2β is degraded following a physiologic stimulus in T cells, the total amount of TRIM27 and PI3KC2β protein was assessed at various time points following TCR stimulation of Jurkat T cells with anti-CD3/CD28 antibodies (Fig. 4B). These findings also failed to demonstrate evidence for degradation of TRIM27 or PI3KC2β following TCR stimulation.

We next assessed whether TRIM27 ubiquitination of PI3KC2β affected PI3KC2β’s kinase activity. GFP-PI3KC2β was cotransfected with or without FLAG-TRIM27(WT) or FLAG-TRIM27(RING MT) and PI3KC2β enzymatic activity was determined on anti-GFP immunoprecipitates. PI3KC2β enzymatic activity was
significantly inhibited in cells cotransfected with TRIM27(WT), resulting in a >60% decrease in PI3KC2β enzymatic activity compared with cells transfected with GFP-PI3KC2β alone (Fig. 4). In contrast, PI3KC2β enzymatic activity was not inhibited, and even slightly increased, in cells transfected with TRIM27 (RING MT). The kinase activity detected was because of PI3KC2β and not an associated kinase, as enzymatic activity was not detected in anti-GFP immunoprecipitates of a kinase dead (KD, K805R).

TRIM27 Negatively Regulates the K⁺ Channel KCa3.1 in Jurkat-KCa3.1 Cells and Primary Human CD4 T Cells by Inhibiting PI3KC2β. Jurkat T cells that overexpress KCa3.1 (Jurkat-KCa3.1) were transfected with an siRNA to TRIM27 and KCa3.1 channel activity was assessed as previously described (19). siRNA knockdown of TRIM27 (Fig. S2A) resulted in about a twofold increase in KCa3.1 channel activity (Fig. S2B–D). The increase in KCa3.1 channel activity was specific and caused by loss of TRIM27’s E3 ligase activity because infection of TRIM27 siRNA transfected cells with a lentiviral construct expressing FLAG-TRIM27(WT), but not FLAG-TRIM27(RING MT), lacking the siRNA binding sequence, restored KCa3.1 channel activity to baseline (Fig. S2D).

To demonstrate that TRIM27 mediates its effect on KCa3.1 by inhibiting PI3KC2β, we assessed whether dialyzing PI3P into Jurkat-KCa3.1 cells transfected with GFP-TRIM27(WT) rescued KCa3.1 channel activity. Overexpression of TRIM27 resulted in a twofold inhibition of KCa3.1 channel activity. The decrease in KCa3.1 channel activity was a result of inhibition of PI3KC2β and decrease in PI3P because KCa3.1 channel activity was rescued by dialyzing GFP-TRIM27 transfected cells with PI3P. Rescue by PI3P was specific because neither PI4P, PI(4,5)P2, nor PI(3,4,5)P3 restored KCa3.1 channel activity in TRIM27 overexpressing cells (Fig. S2F).

TRIM27 Negatively Regulates TCR-Stimulated Ca²⁺ Flux and Cytokine Production in Jurkat-KCa3.1 Cells. Consistent with the increase in KCa3.1 channel activity, TCR-stimulated Ca²⁺ influx was significantly increased in TRIM27 siRNA-transfected Jurkat-KCa3.1 cells (Fig. S3A). In addition, TRIM27 siRNA-transfected Jurkat-KCa3.1 cells secreted about 50% more IL-2 in response to treatment with phorbol myristate acetate and ionomycin (Fig. S3B). The increase in IL-2 production was dependent upon KCa3.1 channel activity because treatment with TRAM34, a specific KCa3.1 inhibitor (38, 39), blocked IL-2 production (Fig. S3B).

KCa3.1 Channel Activity and TCR-Stimulated Ca²⁺ Influx Is Increased in TRIM27−/− Th1 and Th2 CD4 T Lymphocytes. To generate TRIM27−/− mice, the ES cell line 345D11 was purchased from The Center for
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Proximal Signaling Molecules Are Activated Similarly in TRIM27−/− and TRIM27+/+ Th0 Cells. Tyrosine phosphorylation of proximal signaling pathways downstream of the TCR should be unaffected in TRIM27−/− CD4 T cells, if TRIM27 primarily inhibits TCR signaling via direct ubiquitination of PI3KC2β. Anti-CD3/CD28 stimulation resulted in similar levels of total tyrosine phosphorylated proteins in TRIM27−/− and TRIM27+/+ cells (Fig. S6). In addition, activation of AKT and ERK MAP Kinase was similar between TRIM27−/− and TRIM27+/+ cells (Fig. S6).

Discussion

Even though a growing list of stimuli have been shown to activate class II PI3Ks, including chemokines, cytokines, and receptor tyrosine kinases, we still know very little regarding the exact role for class II PI3Ks in mediating biological output from these receptors, or the downstream signaling pathways that are activated (4, 13). We have previously shown that one function of the class II PI3Ks is to generate the pool of PI3P required for activating AKT and ERK MAP Kinase, as well as to identify new signaling molecules that modulate CD4 T-cell activation of NF-κB and MAP Kinase activation (41). TRIM27−/− CD4 T cells were stimulated with anti-CD3/CD28 antibodies (Fig. 6A). Whole-cell patch-clamp experiments demonstrated that KCa3.1 channel activity was increased in TRIM27−/− Th0, Th1 and Th2 CD4 T cells compared with TRIM27+/+ cells, but Shk-sensitive K+ channel activity was similar between the two (Fig. 5 B and C). Consistent with the increase in KCa3.1 channel activity, both the acute rise and the plateau phase of Ca2+ influx was significantly increased in TRIM27−/− Th0, Th1 and Th2 cells following stimulation with anti-CD3 antibodies (Fig. 5 B and C).

Cytokine Production Is Increased in TRIM27−/− CD4 T Cells. To assess whether increased Ca2+ influx leads to increased production of cytokines, TRIM27−/− and TRIM27+/+ CD4 T cells were stimulated with irradiated splenocytes and the superantigen staphylococcal enterotoxin E (SSEE). In comparison with TRIM27+/+ cells, TRIM27−/− cells produced IFN-γ and TNF at lower concentrations of SSEE, and also produced increased amounts of both cytokines at higher SSEE concentrations (Fig. 6A and C). In contrast, production of IL-2 was similar between TRIM27+/+ and TRIM27−/− cells (Fig. 6B).

The TRIM family of proteins are composed of 74 family members in mammalian cells and are divided into 11 subgroups based on their carboxyl-terminal domain (29, 30). A consistent theme that has emerged over the past few years is the important role for TRIM family members in immune activation, and in particular, the regulation of innate immunity (30, 42). For example, TRIM25 promotes K63 ubiquitination of RIG-I, which has been shown to be critical to activate viral innate immunity (43). TRIM30α inhibits toll like receptor (TLR)-mediated activation of NF-κB by targeting TAB2 and TAB3 for degradation (42), and TRIM21 has been shown to inhibit several IFN responses via direct ubiquitination of STAT1 (44). TRIM22 inhibits the TLR4 signalosome and inhibits Stat6 phosphorylation (45). Consistent with the theme that has emerged over the past few years is the important role for TRIM family members in innate immunity, and in particular, the regulation of innate immunity (30, 42). For example, TRIM25 promotes K63 ubiquitination of RIG-I, which has been shown to be critical to activate viral innate immunity (43). TRIM30α inhibits toll like receptor (TLR)-mediated activation of NF-κB by targeting TAB2 and TAB3 for degradation (42), and TRIM21 has been shown to inhibit several IFN responses via direct ubiquitination of STAT1 (44). TRIM22 inhibits the TLR4 signalosome and inhibits Stat6 phosphorylation (45).
Th1, and Th2 cells generated from TRIM27−/− mice (40, 41). Thus, our findings reinforce the critical role for TRIM family proteins to regulate both the innate and adaptive immune response. TRIM27 and other TRIM family members contain an N-terminal Zn-binding RING domain, which enable them to function as E3 ligases (29–31). Although TRIM27 inhibition of IKKε and TBK1 did not require its RING domain (46), TRIM27 inhibition of TCR signaling is mediated via the direct ubiquitination of PI3KC2β by TRIM27. This result is supported by our finding that overexpression of TRIM27(WT), but not a TRIM27(RING MT), inhibited PI3KC2β enzyme activity and rescued the increase in KCa3.1 channel activity following siRNA knockdown of TRIM27. In addition, the ability to rescue TRIM27 inhibition of KCa3.1 channel activity by dialyzing TRIM27 overexpressing cells with PI3P, but not other phosphoinositides, confirms that TRIM27 inhibits KCa3.1 by interfering with PI3P generation. We have previously shown that PI3P is required for KCa3.1 activation by enabling the histidine kinase, NDPK-B, to histidine phosphorylate the C terminus of KCa3.1, leading to its activation (20, 41). Thus, these findings, together with the demonstration that PI3KC2β enzyme activity is increased in TRIM27−/− lymphocytes, supports a model whereby direct ubiquitination of PI3KC2β by TRIM27 results in the inhibition of PI3KC2β’s enzymatic activity leading to decreased levels of PI3P, resulting in decreased histidine phosphorylation and activation of KCa3.1 by NDPK-B (Fig. S7) (19, 40, 41, 47).

Despite the fact that the immune system has evolved a myriad of mechanisms to turn itself off, redundant mechanisms for inhibition are often incomplete. This result is supported by the finding that disruption of even a single pathway is sufficient to lead to autoimmune disease under some circumstances (48). Our finding that TRIM27 is a unique negative regulator of CD4 T cells, when coupled with previous findings that TRIM27 may also negatively regulate innate signaling, places TRIM27 in a unique position to down-regulate the immune response at multiple levels.

Materials and Methods

Cells and Constructs. Jurkat-KCa3.1 T cells (19) and human CD4 T cells were cultured in RPMI + 10% FBS. GFP-tagged PI3KC2α and PI3KC2β were kindly
provided by J. Domín, Imperial College, London, UK. The PI3KC2J2 kinase dead mutant was generated by substituting lysine 850 to arginine and the TRIM27 RING mutant was generated by substituting C31H1/C4C5 in the cysteine-rich zinc binding domain to S3/Q/S4/S5 (33). More details are available in SI Materials and Methods.

In Vivo Ubiquitination Assay. GFP-tagged PI3KC2z or PI3KC2J2 were expressed with or without FLAG-TRIM27 together with His6-ubiquitin in HEK 293 cells. Transfected cells were then lysed in 6 M guanidinium and ubiquitinated proteins were purified using Ni-NTA beads followed by Western blotting with anti-GFP or anti-FAG antibodies, as previously described (49). To assess whether TRIM27 stimulates K48, K63, poly-, or mono-ubiquitination of PI3KC2J2, the Lys48-specific antibody (Millipore; clone Apu2), the poly-ubiquitin-specific antibody (Millipore; clone FK1), or the Lys63-specific anti-body (Millipore; clone Apu3) (50, 51) were used to blot immunoprecipitated PI3KC2J2.

Whole-Cell Patch-Clamp and Intracellular Ca2+ Activity. Whole-cell patch clamping on activated CD4+ T cells (47) and Jurkat-KC3a T cells, PI3P rescue experiment and Ca2+ imaging were performed as previously described (19) and detailed in SI Materials and Methods.

Whole-Cell Patch-Clamp and Intracellular Ca2+ Activity. Whole-cell patch clamping on activated CD4+ T cells (47) and Jurkat-KC3a T cells, PI3P rescue experiment and Ca2+ imaging were performed as previously described (19) and detailed in SI Materials and Methods.

Generation of TRIM27−/− Mice. All procedures were approved by the Institutional Animal Use and Care Committee. ES cell line (clone ID 345D11, Biochem Cell Trans) containing exon-trapping plasmid pUPA integrated between exon 1 and exon 2 of the TRIM27 gene was purchased from The Center for Modeling Human Disease at the University of Toronto (http://www.cmhd.ca/genetrap/index.html). ES cells (strain 129/oLa) were injected into C57Bl6/blastocyst by the transgenic facility at the New York University Langone Medical Center (New York) and chimeric mice were obtained. TRIM27−/− mice were backcrossed six generations with C57Bl6 and then used to generate TRIM27−/+ mice in these studies. CD4+ T cells were purified on MACS beads (Miltenyi Biotech) from WT or TRIM27−/− spleens and various CD4 T-cell subsets were generated, as previously described (52).

Cytokine Assays. For cytokine assays, TRIM27−/− and TRIM27+/+ CD4 Th0 cells were stimulated for 3 d together with splenocytes in the presence of various concentrations of SE. Cytokines were assayed in supernatants using the BD Cytometric Bead Array Cytokine Kit.

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Supporting Information

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SI Materials and Methods

Cells and Constructs. Jurkat-KCa3.1 T cells were generated as previously described (1) and cultured in RPMI + 10% FBS. GFP-tagged class II PI3K C2α (PI3KC2α) and PI3KC2β were kindly provided by J. Domin, Imperial College, London, United Kingdom. The PI3KC2β kinase dead mutant was generated by substituting lysine 850 to arginine and tripartite motif containing protein 27 (TRIM27) Really Interesting New Gene (RING) mutant was generated by substituting C5/H1/C4/CS in the cysteine-rich zinc binding domain to S3/Q1/S4/S5 (2).

All T-cell transfection were performed using AMAXA reagents according to the manufacturer’s protocols (Amaza Biosystems). Human CD4 T cells were isolated from peripheral adult blood buffy coats (New York Blood Center) using the CD3 isolation kit from Miltenyi Biotec according to manufacturer’s protocol. We routinely obtained >95% CD4 T cells as assessed by FACS.

Antibodies. Anti-TRIM27 antibodies were purchased from IBL America and anti-PI3KC2β were purchased from BD Transduction Laboratories. Anti-PI3KC2β antibody M02 (clone 3E5) (Novus Biologicals) was used to both immunoblot and immunoprecipitate mouse PI3KC2β.

In Vivo Ubiquitination Assay. GFP-tagged PI3KC2α and PI3KC2β were expressed with or without FLAG-TRIM27 together with Hisβ-ubiquitin in HEK 293 cells. Transfected cells were then lysed in 6 M guanidinium and ubiquitinated proteins were purified using Nickel (Ni)-NTA beads followed by Western blotting with anti-GFP or anti-FLAG antibodies, as previously described (3).

To assess whether TRIM27 stimulates K48, K63, poly-, or monoubiquitination of PI3KC2β, HEK 293 cells were transfected with GFP-PI3KC2β and FLAG-TRIM27. Twenty-four hours later, cells were lysed in 1% SDS lysis buffer and, after diluting to 0.1% SDS, PI3KC2β was immunoprecipitated with anti-GFP antibodies and then Western blotted with the Lys48-specific antibody (Millipore; clone Apu3), the polyubiquitin-specific antibody (Millipore; clone FK1), of the Lys63-specific antibody (Millipore; clone Apu3), as previously described (4, 5).

Whole-Cell Patch Clamp. CD4 T cells. Whole-cell patch clamping was performed on activated CD4 T cells 48 h after stimulation with anti-CD3 and antiCD28 antibodies. Jurkat-KCa3.1 T cells was performed as previously described (6), with some modification (7).

Jurkat-KCa3.1 T cells. Whole-cell patch clamping on Jurkat-KCa3.1 T cells was performed as previously described (1). To verify that TRIM27 modulated KCa3.1 channel activity via PI3KC2β, which resulted in changes in levels PI3P, PI3P (100 nM) was added into the pipette solution during patch clamping in Jurkat-KCa3.1 cells overexpressing GFP-TRIM27 (8). PI(3)P and stimulated with 5 μM Fura-2/AM ester (Molecular Probes) in RPMI medium for 30 min at room temperature, washed, and then resuspended in RPMI. Cells were attached to poly(l)lysine-coated coverslips for 20 min in a RC-20 bath flow chamber (Warner Instrument) and fura-2 fluorescence was recorded (Delta Ram; PFI Inc.) at excitation wavelengths of 340 and 380 nm. Background fluorescence was obtained by treating the cells with 100 mM MnCl2 in the end of the experiment. Cells are represented as the ratio 340/380 after background subtraction. Cells were perfused with the bath solution (composition described before) in the presence or absence of extracellular Ca2+ and stimulated with 5 μg/mL of anti-CD3 cross linked with 5 μg/mL of rat anti-mouse IgG.

PI3K Assay. HEK 293 cells were transfected with GFP-PI3KC2β WT or kinase dead mutant alone or together with TRIM27 (WT) or TRIM27 (RING MT). Cells were lysed in the presence of proteinase inhibitors, phosphatase inhibitors, and p5 μM ubiquitin-aldehyde (Boston Biochem), and PI3K assay was performed on anti-GFP immunoprecipitates, as previously described (1). Lymphocytes from TRIM27+/− and TRIM27−/− mice were lysed as above and PI3K assay was performed on anti-PI3KC2β (Novus Biologicals) immunoprecipitates, also as previously described (1).

Generation of TRIM27−/− Mice. ES cell line (clone ID 345D11, strain 129/ola) that contains an exon-trapping plasmid pUPA integrated between exon 1 and exon 2 of the TRIM27 gene was purchased from The Center for Modeling Human Disease at the University of Toronto. (http://www.cmhd.ca/genetrap/index.html). ES cells (strain 129/ola) were injected into C57BL/6 blastocyst by the transgenic facility at the New York University Langone Medical Center (New York) and chimeric mice were obtained. TRIM27+/− mice were backcrossed five generations with C57BL/6 and then used to generate TRIM27−/− C57BL/6 mice in these studies.

Primers used to genotype mice: TRIM27 F WT (mouse chromosome 13 genomic contig, GenBank Accession Number NT_039578.7, 10099908–10099931) CATTAGCCACTCTGACTCGGG; TRIM27 R (mouse chromosome 13 genomic contig, GenBank Accession Number NT_039578.7, 10100140–10100166). AGGAGTGAGATAACAGTGACAGC; pUPA F KO GAT-AAGTGTGCTGGCCAGCTTACCTCCC. The WT PCR product (primers TRIM27 F WT and TRIM27 R) is 259 bp and the knockout PCR product (primers pUPA F KO and TRIM27 R) is 600 bp.

Purification and Differentiation TRIM27−/− and TRIM27−/− CD4 T Subsets. CD4+ T cells were purified on MACS beads (Miltenyi Biotech) from WT or TRIM27−/− spleens as previously described (11). Various CD4 T-cell subsets were generated by

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culturating CD4 cells purified from spleen cells under Th1 polarizing conditions (100 U/mL IL-12 and anti-IL-4) or Th2 polarizing conditions (100 U/mL IL-4, anti–IFN-γ) for 4–6 d.

**Quantitative Real-Time PCR.** Total RNA was isolated using TRIzol reagent and then reverse transcribed using random hexamer primers. Quantitative PCR was then assessed using SYBR Green 1 by iCycler iQ (BioRad) using gene specific primers purchased from Qiagen.

**Cytokine Assays.** For cytokine assays, TRIM27\(^{-/-}\) and TRIM27\(^{+/+}\) CD4 T cells were stimulated for 48 h with anti-CD3/CD28 antibodies and, after resting overnight, were restimulated for 3 d together with splenocytes in U-bottom 96-well plates at a ratio of 1:8 (T cells:splenocytes) in the presence of various concentrations of staphylococcal enterotoxin E (SEE). Cytokines were assessed in supernatants using the BD Cytometric Bead Array Cytokine Kit.


**Fig. S1.** TRIM27 partially colocalizes with the transferrin receptor. COS cells expressing GFP-TRIM27 were incubated with rhodamine-transferrin (trf), lysotracker, and mitotracker (Molecular Probes), and colocalization was assessed by fluorescence. Magnification, 60x.
Fig. S2. TRIM27 negatively regulates KCa3.1 channel activity in Jurkat T cells overexpressing KCa3.1 (Jurkat-KCa3.1) and human CD4 T cells. siRNA knockdown of TRIM27 in Jurkat-KCa3.1 cells was assessed by (Ai) Western blot and (ii) real-time PCR. The Western blot in Ai was reprobed with β-actin to demonstrate equal loading between lanes, and the results in Aii show the relative amount of TRIM27 expression standardized to a GAPDH control \( n = 4 \). Whole-cell patch clamp of (B) control or (C) TRIM27 siRNA-transfected cells. (D) Bar graph summary of TRAM34 inhibited currents plotted at +60 mV from Jurkat-KCa3.1 cells transfected with ctrl or TRIM27 siRNA shown in B and C. Shown are the average from 10 cells per condition. *\( P < 0.001 \), TRIM27 siRNA knockdown cells rescued with either FLAG-TRIM27(WT) and FLAG-TRIM27(RING MT). (E) Purified human CD4 cells were transfected with an siRNA to TRIM27 and then stimulated with anti-CD3 and CD28 antibodies for 48 h. Shown are the bar graph summary of TRAM34 (KCa3.1) and Shk (Kv1.3) inhibited currents plotted at +60 mV. (F) PI3P (100 nM) was added into the pipette solution during patch clamping in Jurkat-KCa3.1 cells overexpressing GFP-TRIM27. Plotted are means ± SE from at least three independent experiments. Also shown in F is the failure of PI4P, PI(4,5)P₂, or PI(3,4,5)P₃ to rescue. *\( P < 0.05 \).
Fig. S3. Increased Ca\textsuperscript{2+} influx and IL-2 production following siRNA knockdown of TRIM27 in Jurkat-KCa3.1 cells. (A) Cells in Fig. S2 were loaded with Fura-2:00 AM (5 mM) and Ca\textsuperscript{2+} flux was determined after cross-linking with anti-CD3 antibodies. (B) Control or TRIM27 siRNA-transfected cells were cultured with phorbol myristate acetate/ionomycin, and IL-2 secretion in the supernatant was determined 24 h after stimulation in the absence or presence of TRAM34, as described. *P < 0.05.

Fig. S4. Generation of TRIM27\textsuperscript{−/−} mice. (A) Schematic representation of the TRIM27 gene of clone 345D11 showing the insertion of pUPA in the intron between exons 1 and 2 of the TRIM27 gene. (B) To amplify the WT TRIM27 allele, oligonucleotides were synthesized that were complementary to genomic DNA on either side of the insertion site of pUPA and used to amplify the WT locus by PCR (bp 259). The mutant TRIM27 locus was amplified by PCR using the same 3′ oligo used to amplify the WT locus and an oligo close to the 3′ end pUPA (bp 600). (C) Total lysates were isolated from spleen and thymus of TRIM27\textsuperscript{−/−} and TRIM27+/+ mice and Western blotted with antibodies to TRIM27 and PI3KC2\textbeta. The same blot was then reprobed with antibodies to β-actin to demonstrate equal loading of protein in both lanes. (Di) Lymphocytes from TRIM27\textsuperscript{−/−} and TRIM27\textsuperscript{+/+} mice were lysed and PI3K assay was performed on anti-PI3KC2\textbeta immunoprecipitates. *P < 0.05. (ii) Fifty percent of the immunoprecipitate in Di was immunoblotted with anti-PI3KC2\textbeta antibodies to demonstrate equal amounts of PI3KC2\textbeta protein in the two samples. Lysates were also immunoblotted with anti-TRIM27 antibodies.
Fig. S5. T- and B-cell development is normal in TRIM27−/− mice. Cells were isolated from spleen, thymus, and lymph node (LN) from WT and TRIM27−/− mice and stained with antibodies to (A) CD3 and CD19, (B) CD4 and FoxP3, or (C) CD4 and CD8 followed by FACS analysis. All experiments shown are representative of at least four experiments performed on cells isolated from at least four pairs of independent mice.

Fig. S6. Activation of T-cell receptor (TCR) stimulation of proximal signaling pathways is similar between TRIM27−/− and WT Th0 cells. TRIM27−/− and WT Th0 cells were stimulated with anti-CD3/CD28 antibodies for various times and Western blotted with antibodies to phosphotyrosine (pY), phospho-AKT (pAKT), or phospho-ERK (pERK1/2).
Fig. S7. Model for TCR-stimulated activation of KCa3.1 mechanism for regulation by TRIM27. Activation of two signaling pathways is required for TCR-stimulated activation of KCa3.1 and T-cell activation. Signal 1: TCR activation of PLCγ results in the generation of IP$_3$, stimulating release of Ca$^{2+}$ from the endoplasmic reticulum, opening of calcium release-activated Ca$^{2+}$ channels (CRAC), and the influx of Ca$^{2+}$; signal 2: TCR stimulation also activates PI3KC2β leading to the generation of PI(3)P, which is required for nucleoside diphosphate kinase B (NDPK-B) to phosphorylate histidine 358 in the carboxy-terminus (CT) of KCa3.1. Binding of Ca$^{2+}$ to the calmodulin bound to the CT of KCa3.1 and phosphorylation of H358 in CT of KCa3.1 by NPDK-B is required for KCa3.1 activation. TRIM27 inhibits signal 2 by ubiquitinating and inhibiting PI3KC2β's activity.