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T Cell Receptor-Interacting Molecule Acts as a Chaperone to Modulate Surface Expression of the CTLA-4 Coreceptor

Elke Valk,[1,2] Rufina Leung,[2] Hyun Kang,[3] Kazuyo Kaneko,[2] Christopher E. Rudd,[1,2] and Helga Schneider[1,2,*]

[1] Cell Signalling Section
Division of Immunology
Department of Pathology
Cambridge University
Cambridge CB2 1QP
United Kingdom

[2] Molecular Immunology Section
Department of Immunology
Division of Investigative Science
Faculty of Medicine
Imperial College London
Hammersmith Campus
London W12 ONN
United Kingdom

[3] Department of Medicine
Harvard University
Cambridge, Massachusetts 02115

Summary

The costimulatory molecule CTLA-4 is a potent downregulator of T cell responses. Although localized mostly in intracellular compartments, little is understood regarding the mechanism that regulates its transport to the cell surface. In this study, we demonstrated that the adaptor TRIM (T cell receptor-interacting molecule) bound to CTLA-4 in the *trans* Golgi network (TGN) and promoted transport of CTLA-4 to the surface of T cells. Increased TRIM expression augmented surface CTLA-4 expression, and pulse-chase analysis showed a more rapid transport of CTLA-4 to the cell surface. A reduction of TRIM expression by small hairpin RNAs reduced the expression of surface CTLA-4. This resulted in a more localized pattern of CTLA-4 in the TGN. Altered CTLA-4 expression by TRIM was accompanied by corresponding changes in coreceptor-mediated effects on cytokine production and proliferation. Our findings identify a role for TRIM as a chaperone in regulating CTLA-4 expression and function by enhancing CTLA-4 transport to the surface of T cells.

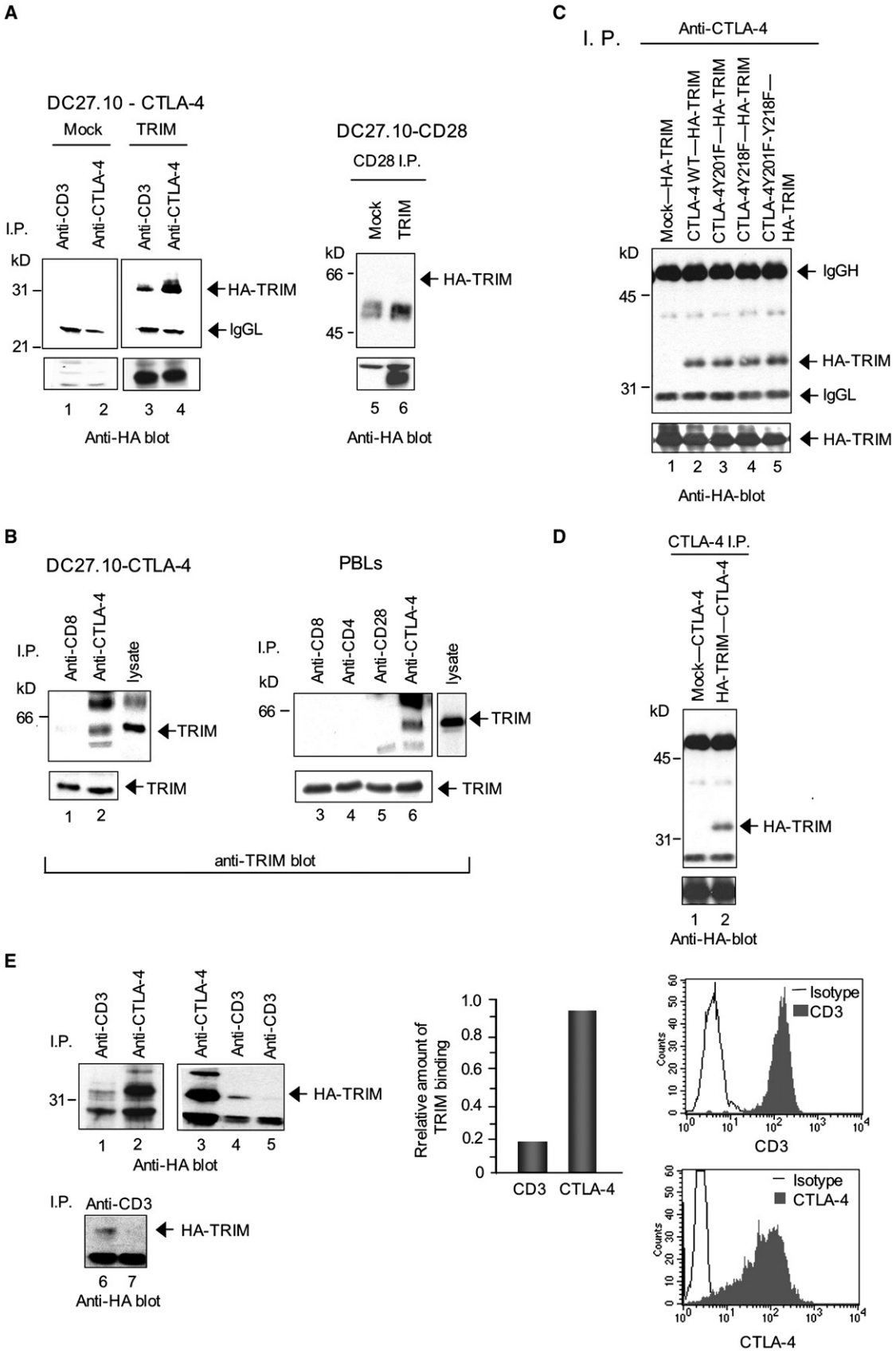
Introduction

The costimulatory molecules CD28 and CTLA-4 determine the ultimate outcome of the immune response after ligation of the T cell receptor (TCR)–CD3 complex. Both bind to CD80 and CD86 and are required for optimal T cell activation (Bluestone, 1995; Linsley, 1995; Thompson, 1995). In contrast to CD28, which enhances and sustains T cell responses, CTLA-4 potently inhibits T cell activation (Krummel and Allison, 1995; Linsley, 1995; Walunas et al., 1994). CTLA-4-deficient (*Ctla-4*$^{-/-}$) mice show an autoimmune phenotype with organ destruction (Tivol et al., 1995; Waterhouse et al., 1995). Various mechanisms have been proposed to account for the molecular mechanism by which CTLA-4 generates inhibitory signals. Reported mechanisms include ectodomain competition for CD28 binding to CD80 and CD86 (Masteller et al., 2000), disruption of CD28 localization at the immunological synapse (Pentcheva-Hoang et al., 2004), modulation of phosphatases PP2A and SHP-2 (Chuang et al., 2000; Lee et al., 1998; Marengere et al., 1996), and interference with lipid raft expression (Chikuma et al., 2003; Darlington et al., 2002; Martin et al., 2001; Rudd et al., 2002). CTLA-4 engagement of CD80 and CD86 on dendritic cells can also induce the release of indoleamine 2,3-dioxygenase (IDO) (Boasso et al., 2004; Fallarino et al., 2003). In addition, we have demonstrated that CTLA-4 is a potent inducer of integrin adhesion, and can reverse the stop signal needed for stable T cell–APC conjugation (Schneider et al., 2005, 2006).

One of the unusual characteristics of CTLA-4 is that it is primarily located in intracellular compartments (Alegre et al., 1996; Leung et al., 1995). Little of the coreceptor can be detected on the surface of cells, even during its optimal expression after T cell activation. This tight regulation is presumably due to the need to control surface expression of this potent coreceptor. Two pools of intracellular CTLA-4 have been described, in the *trans* Golgi apparatus (TGN) and in endo- and lysosomal compartments (Alegre et al., 1996; Iida et al., 2000; Leung et al., 1995; Linsley et al., 1996). TCR ligation and calcium ionophores can induce CTLA-4 release to the cell surface; however, the mechanism is unknown (Linsley et al., 1996). The other event known to affect CTLA-4 expression involves the mutation of the YVKM motif in CTLA-4, which can increase expression mediated by impaired internalization via the clathrin adaptor complex AP-2 (Yi et al., 2004). Nonphosphorylated CTLA-4 can bind to AP-1 and AP-2 tetramers that regulate trafficking and endocytosis of CTLA-4, respectively (Bradshaw et al., 1997; Chuang et al., 1997; Schneider et al., 1999; Shiratori et al., 1997; Zhang and Allison, 1997). AP-1 binding to CTLA-4 mediates its transport from the Golgi apparatus to the lysosomal compartment for degradation (Schneider et al., 1999). Once on the cell surface, other mechanisms (i.e., binding to AP-2) regulate its endocytosis (Shiratori et al., 1997; Zhang and Allison, 1997). Phosphorylation of CTLA-4 by the kinases p56lck, p59fyn, and Rlk can inhibit these events (Bradshaw et al., 1997; Chuang et al., 1997; Miyatake et al., 1998; Schneider et al., 1998).

Adaptor proteins are essential for precise signal transduction by allowing the formation of signaling scaffolds (Rudd, 1999; Samelson 2002). Two main groups of adaptor proteins can be distinguished: cytosolic and transmembrane adaptor proteins. An array of transmembrane adaptor proteins (TRAPs) has been identified including LAT (linker for activation of T cells), LIME (lck interacting membrane protein), TRIM (T cell receptor-interacting molecule), SIT (SHP2 interacting transmembrane adaptor protein), PAG/Cbp (protein associated

*Correspondence: hs383@cam.ac.uk


A
DC27.10 - CTLA-4
Mock
TRIM
Anti-CD3
Anti-CTLA-4
Anti-CD3
Anti-CTLA-4
I.P.
kD
31
21
HA-TRIM
IgGL
1 2 3 4
Anti-HA blot
DC27.10-CD28
CD28 I.P.
Mock
TRIM
kD
66
45
HA-TRIM
5 6
Anti-HA blot
C
I. P.
Anti-CTLA-4
Mock—HA-TRIM
CTLA-4 WT—HA-TRIM
CTLA-4Y201F—HA-TRIM
CTLA-4Y218F—HA-TRIM
CTLA-4Y201F-Y218F—HA-TRIM
kD
45
31
IgGH
HA-TRIM
IgGL
HA-TRIM
1 2 3 4 5
Anti-HA-blot
B
DC27.10-CTLA-4
PBLs
I.P.
Anti-CD8
Anti-CTLA-4
lysate
Anti-CD4
Anti-CD28
kD
66
TRIM
1 2 3 4 5 6
anti-TRIM blot
D
CTLA-4 I.P.
Mock—CTLA-4
HA-TRIM—CTLA-4
kD
45
31
HA-TRIM
1 2
Anti-HA-blot
E
I.P.
Anti-CD3
Anti-CTLA-4
Anti-CTLA-4
Anti-CD3
Anti-CD3
31
HA-TRIM
1 2 3 4 5
Anti-HA blot
I.P. Anti-CD3
HA-TRIM
6 7
Anti-HA blot
Rrelative amount of TRIM binding
1.0
0.8
0.6
0.4
0.2
0
CD3
CTLA-4
Isotype
CD3
Counts
CD3
Isotype
CTLA-4
Counts
CTLA-4

with GEMs/Csk binding protein), and LAX (linker for activation of X) (Kliche et al., 2004).

Of these, TRIM (official gene name *trat1*) is a 30 Kd type III transmembrane protein, which is expressed in human T lymphocytes and NK cells (Bruyns et al., 1998; Kirchgessner et al., 2001). It comprises a short 8 amino acid extracellular domain, a 19 amino acid transmembrane region, and a 159 amino acid cytosolic tail. Its cytoplasmic tail contains several tyrosine motifs with the potential to bind to Src-homology 2 (SH2) domains of signaling proteins. Upon TCR stimulation, TRIM becomes rapidly tyrosine phosphorylated and associates with the p85 subunit of the lipid kinase PI3-kinase (Bruyns et al., 1998). Association of TRIM with the TCRζ-CD3 complex has been shown in Jurkat T cells and human peripheral blood lymphocytes (PBLs) (Bruyns et al., 1998). Overexpression of TRIM in Jurkat T cells has been reported to induce an increase in TCR surface expression, although this does not occur in primary T cells (Kirchgessner et al., 2001; Kölsch et al., 2006).

Given the importance of CTLA-4 to T cell immunity, a major outstanding question concerns the mechanism by which the coreceptor is shuttled to the cell surface to downregulate the immune response. In this study, we demonstrate that TRIM facilitates shuttling of CTLA-4 from the TGN to the surface of T cells. The adaptor was found to bind and colocalize with CTLA-4 in the TGN. TRIM overexpression increased CTLA-4 expression and its ability to inhibit T cell proliferation and interleukin-2 (IL-2) production. Moreover, the reduction of TRIM expression by shRNA reduced CTLA-4 cell-surface expression and CTLA-4-mediated inhibition of cytokine release. Further, subcellular distribution of CTLA-4 was altered in TRIM knockdown cells. Together, our findings identified a role for TRIM as a chaperone in modulating CTLA-4 surface expression and thus regulating T cell function.

Results

TRIM Binds to CTLA-4 and CD3, but Not to CD28

The transmembrane adaptor protein was initially identified as a protein that coprecipitates and comodulates the TCR–CD3 complex (Bruyns et al., 1998; Kirchgessner et al., 2001). TRIM was reported to specifically associate with the TCRζ chain, but not with coreceptors such as CD28, CD2, CD4, and CD8 (Bruyns et al., 1998). However, it was not known whether TRIM binds to other receptors. In order to assess whether TRIM associates with CTLA-4, DC27.10 hybridoma T cells expressing CTLA-4 (DC27.10-CTLA-4) were initially transfected with HA-tagged TRIM and subjected to immunoprecipitation with anti-CD3 or anti-CTLA-4. Anti-CTLA-4 readily coprecipitated TRIM when compared to mock transfected cells (Figure 1A). This binding was considerably higher than seen with CD3. Further, TRIM bound selectively to CTLA-4 but not to CD28 (Figure 1A; Bruyns et al., 1998) as shown by DC27.10 hybridoma T cells expressing CD28 (DC27.10-CD28) transfected with HA-tagged TRIM or mock and immunoprecipitated with anti-CD28.

To assess the binding of endogenous TRIM to CTLA-4, DC27.10-CTLA-4 cells and human PBLs were lysed and subjected to precipitation (Figure 1B). Anti-CD8, anti-CD4, anti-CD28, and anti-CTLA-4 immunoprecipitates were subjected to blotting for endogenous TRIM with anti-TRIM under nonreducing condition. Although no binding of endogenous TRIM to CD4, CD8, and CD28 was observed, endogenous TRIM was readily coprecipitated with CTLA-4 in DC27.10-CTLA-4 cells and human PBLs. These observations confirm that endogenous TRIM and CTLA-4 interact in primary T cells.

CTLA-4 interacts with the lipid kinase phosphatidylinositol 3-kinase (PI3-K) via SH2 domain binding to the YVKM motif (Schneider et al., 1995). Mutation of the Y-201 residue disrupts PI3-K binding (Schneider et al., 1999). Similarly, AP-1 and AP-2 binding was abrogated in the CTLA-4-Y201F mutant (Schneider et al., 1999). To assess whether TRIM binding to CTLA-4 could occur independently of the binding of PI3-K and the AP-1 and AP-2 complexes, anti-CTLA-4 was used to precipitate TRIM from cells expressing wild-type and the CTLA-4 Y201F mutant (Figure 1C). Under these conditions, TRIM was coprecipitated at equal amounts from wild-type and Y201F mutant cells. In addition, mutation of the two tyrosines Y201 and Y218 in CTLA-4 cytoplasmic tail had no effect on the binding of TRIM to CTLA-4. These observations demonstrated that TRIM binds to CTLA-4 independently of PI3-K and AP-1 and AP-2 complex binding.

As a further control, it was also important to assess whether other lymphoid components such as the TCRζ-CD3 complex were involved in the CTLA-4–TRIM

Figure 1. TRIM Binds to CD3 and CTLA-4, but Not CD28

(A) DC27.10-CTLA-4 (lanes 1–4) and DC27.10-CD28 cells (lanes 5, 6) transfected with mock (lanes 1, 2, 5) or HA-TRIM (lanes 3, 4, 6) were immunoprecipitated with anti-CTLA-4 (lanes 2, 4), anti-CD3 (lanes 1, 3), and anti-CD28 (lanes 5, 6; nonreducing condition) and immunoblotted with anti-HA (lanes 1–6). Expression of HA-TRIM is shown in cell lysates immunoblotted with anti-HA (bottom). The results are representative of three independent experiments.

(B) DC27.10-CTLA-4 cells (left) and PBLs (right) were precipitated with anti-CD8 (lanes 1, 3), anti-CD4 (lane 4), anti-CD28 (lane 5), or anti-CTLA-4 (lanes 2, 6) and blotted for endogenous TRIM (lanes 1–6, nonreducing condition). The lower panel shows the expression of endogenous TRIM in cell lysate. Similar results were obtained from another independent experiment.

(C) COS cells cotransfected with HA-TRIM and either mock (lane 1), CTLA-4 WT (lane 2), CTLA-4 Y201F mutant (lane 3), CTLA-4 Y218F mutant (lane 4), and CTLA-4Y201F-Y218F mutant (lane 5) were precipitated with anti-CTLA-4 and blotted with anti-HA. Expression level of HA-TRIM in cell lysates is shown in the lower panel.

(D) COS cells cotransfected with mock and CTLA-4 (lane 1) or HA-TRIM and CTLA-4 (lane 2) were precipitated with anti-CTLA-4 and blotted with anti-HA (lanes 1, 2). Expression of HA-TRIM is shown in cell lysates immunoblotted with anti-HA (bottom).

(E) HA-TRIM-transfected DC27.10-CTLA-4 cells were either first precipitated with anti-CD3 (lane 1) and, after preclearing, subjected to anti-CTLA-4 precipitation (lane 2), or first precipitated with anti-CTLA-4 (lane 3), followed by two anti-CD3 precipitations (lanes 4, 5) and blotted for HA-TRIM. Lanes 6 and 7 show CD3 depletion by one anti-CD3 precipitation from TRIM-transfected cells. Densitometric analysis of CTLA-4 and CD3-precipitated amounts of TRIM is shown in the middle panel. Right: DC27.10-CTLA-4 cells were stained for CD3 or CTLA-4 and analyzed by flow cytometry. Results in (C)–(E) are representative of three independent experiments.

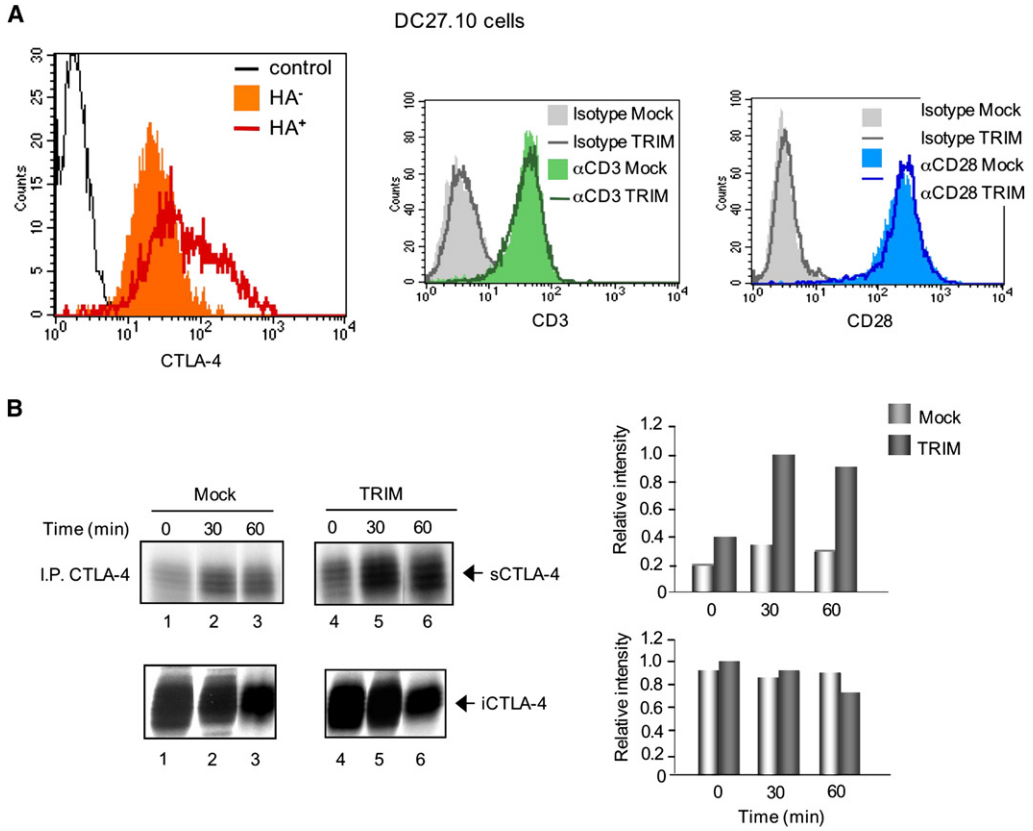


Figure 2. TRIM Transfection Leads to Increased CTLA-4 Surface Expression

(A) DC27.10-CTLA-4 and DC27.10-CD28 cells were transfected with TRIM or mock, respectively. After 24 hr, cells were stained with anti-CTLA-4. Subsequently, cells were fixed with 4% PFA, permeabilized with saponin, and stained for HA-TRIM with anti-HA. HA-negative and HA-positive cells were gated for analysis. Additionally, cells were stained for CD3 and CD28 expression, with CD3 FITC or CD28 PE-labeled mAbs, respectively (bottom). Results are representative of at least five experiments.

(B) To monitor CTLA-4 surface expression of newly synthesized CTLA-4 in mock (lanes 1–3) or TRIM (lanes 4–6)-transfected DC27.10-CTLA-4 cells, cells were pulsed with [^{35}S]methionine for 20 min and chased with nonradioactive methionine for the indicated periods of time in the presence of ionomycin (1 μM). Surface CTLA-4 (sCTLA-4) from both transfectants was precipitated by preincubating labeled cells with anti-CTLA-4 at 4°C, followed by washing and immunoprecipitation. Lysates were subjected to a round of preclearing with Protein A-Sepharose, followed by a second precipitation to purify intracellular CTLA-4 (iCTLA-4). The precipitates were analyzed by 10% SDS-PAGE (left); densitometric measurement of intracellular (bottom) and surface (top) CTLA-4, relative to maximum signal, is shown in the right panel. Similar results were obtained from two other independent experiments.

binding. As mentioned, TRIM has previously been reported to associate with the TCRζ-CD3 complex on T cells (Bruyns et al., 1998; Kirchgessner et al., 2001). For this, CTLA-4 and HA-TRIM were coexpressed in COS cells, which are nonlymphoid cells, and assessed for binding (Figure 1D). Anti-CTLA-4 coprecipitated TRIM from TRIM-transfected, but not from mock transfected, cells. This indicated that TRIM could bind to CTLA-4 independently of the TCRζ-CD3 complex and other lymphoid components. To compare the amount of TRIM binding to the TcRζ-CD3 complex versus CTLA-4, an initial precipitation was carried out with anti-CD3 followed, after preclearing, by a second precipitation with anti-CTLA-4 from the CD3-depleted lysate. Given the normal limitations of different antibody affinities, we observed about five times more TRIM associated with CTLA-4 than with TcRζ-CD3 (Figure 1E). This was also observed when the first precipitation was done with anti-CTLA-4 and, after preclearing, with anti-CD3. An additional anti-CD3 precipitation was ineffective as also shown with sequential anti-CD3 precipitations only. This higher amount of binding of CTLA-4 to TRIM occurred even in the presence of 1% Triton X-100 and occurred under conditions where the expression levels of TCRζ-CD3 and CTLA-4 were comparable (Figure 1E).

Thus, CTLA-4 precipitated TRIM in the absence of TcRζ-CD3, and the amount of CTLA-4-associated TRIM was higher than observed with CD3–TRIM.

TRIM Upregulates CTLA-4 Surface Expression

The CTLA-4–TRIM interaction and their intracellular localization suggested that TRIM might regulate the surface expression of the coreceptor. The mechanism that regulates CTLA-4 has been a major question in CTLA-4 biology with implications to the regulation of T cell function. Previous studies have implicated the Y201 residue in controlling the expression of surface CTLA-4 (sCTLA-4) (Yi et al., 2004; Leung et al., 1995). CTLA-4 expression was assessed on the surface of DC27.10-CTLA-4 cells that had been transfected with HA-TRIM (Figure 2A). Expression of HA-TRIM increased the mean fluorescence intensity of sCTLA-4 expression.

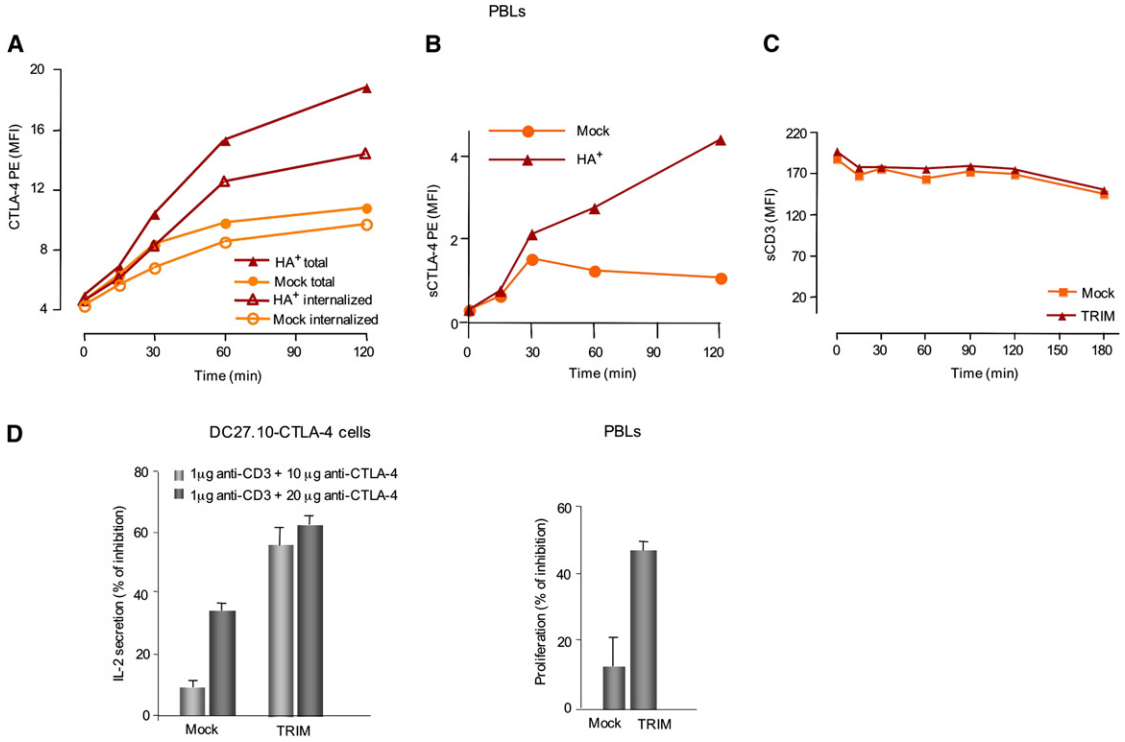


Figure 3. Trim Upregulates CTLA-4 Surface Expression in Human PBLs and Enhances Inhibition of CD3-Mediated Proliferation and IL-2 Production by CTLA-4

(A) Preactivated human peripheral T cells were transfected with TRIM or mock with Amaxa nucleofection. TRIM- or mock transfected PBLs were stimulated with 1 μM ionomycin for up to 2 hr in the presence of anti-CTLA-4. Cells were fixed, permeabilized, and stained for HA-TRIM. HA-TRIM-expressing cells were gated, and total cell-associated PE fluorescence representing surface and internalized CTLA-4 was analyzed in HA-FITC-positive cells compared to the mock transfected sample. To determine internalized CTLA-4, cells were treated with PBS (pH 2) before HA-TRIM staining. Total and internalized CTLA-4-PE fluorescence is shown.

(B) The difference between total CTLA-4 staining and internalized material representing CTLA-4 surface staining is shown.

(C) TRIM- or mock transfected PBLs were stimulated with ionomycin (1 μM) for up to 3 hr. At the indicated time points, surface CD3 (sCD3) expression was analyzed with anti-CD3. Results in (A)–(C) are representative of four independent experiments.

(D) TRIM- or mock transfected DC27.10-CTLA-4 cells (left) were stimulated with plate-bound mAbs for 24 hr (anti-CD3, 1 μg/ml; anti-CTLA-4, 10 μg/ml or 20 μg/ml) as indicated. Supernatants were analyzed for IL-2 by ELISA. Percentage of inhibition in anti-CD3- and anti-CTLA-4-treated cells was calculated in comparison to anti-CD3-stimulated cells. TRIM- or mock transfected PBLs (right) were stimulated with plate-bound mAbs for 24 hr (anti-CD3 2 μg/ml, anti-CTLA-4 20 μg/ml) and mitomycin C-treated APCs (5:1) as indicated. [^{3}H]thymidine was added after 24 hr and [^{3}H]thymidine incorporation was measured. Percentage of inhibition in anti-CD3- and anti-CTLA-4-treated cells was calculated in comparison to anti-CD3-stimulated cells. Bar graphs show mean ± SD. Similar results were obtained from at least two other independent experiment.

In contrast, TCR-CD3 and CD28 expression was not affected. These findings indicate that an increase in TRIM expression can increase the expression of sCTLA-4.

In another approach, pulse-chase analysis was used to track CTLA-4 from intracellular compartments to the cell surface. Mock and TRIM-transfected cells were pulsed with [^{35}S]methionine for 20 min followed by a chase with nonradioactive methionine for 30–60 min. Surface CTLA-4 from both transfectants was then precipitated by preincubating the labeled cells with anti-CTLA-4, followed by washing and immunoprecipitation (Figure 2B). The remaining material was then subjected to a second precipitation in order to precipitate the remaining intracellular CTLA-4 (iCTLA-4). Under these conditions, it was evident that pulse-labeled CTLA-4 appeared more quickly and at higher amounts on the surface of T cells in TRIM-transfected cells. The difference in CTLA-4 surface expression between mock and TRIM transfectants was most prominent after 30 and 60 min stimulation (in average three times greater than in mock transfectants). Even with TRIM expression, the amount of sCTLA-4 comprised only a small portion of total CTLA-4 in cells. Overall, these findings indicate that the mere expression of TRIM increased the rate and amount of sCTLA-4 released from intracellular compartments.

TRIM Leads to Enhanced CTLA-4 Cycling

Having established that TRIM increased the transport of CTLA-4 to the cell surface, it was also important to determine whether the increase in sCTLA-4 was accompanied by increased receptor internalization. For this, CTLA-4 was labeled with anti-CTLA-4-PE, continuously present in the medium, during stimulation by ionomycin (Figure 3) or anti-CD3 (data not shown) of primary T cells. Total cell-associated PE-fluorescence representing surface and internalized CTLA-4 was analyzed at different times. Consistent with the pulse-chase analysis, total cell-associated PE-fluorescence was higher in HA-TRIM-transfected cells than in mock transfectants

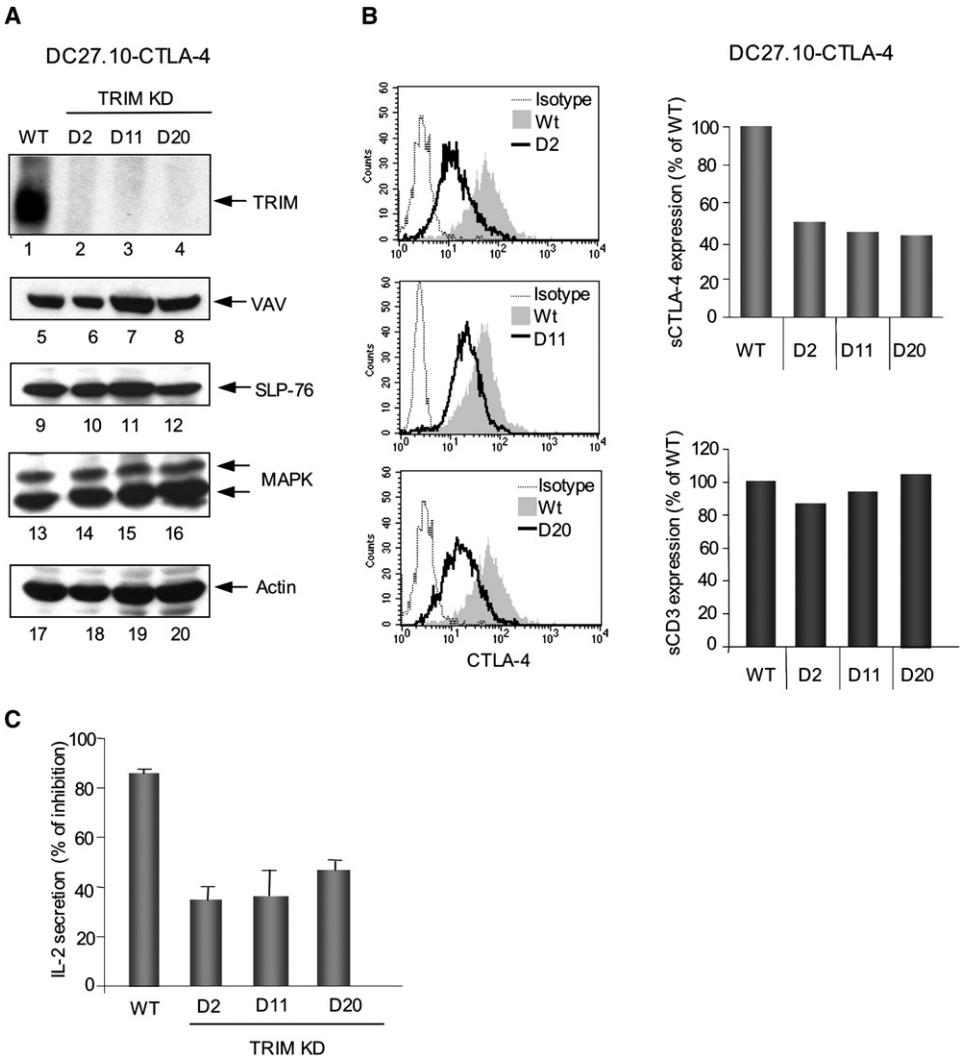


Figure 4. Downregulation of TRIM by shRNA Leads to Impaired CTLA-4 Surface Expression and Inhibition of CD3-Mediated IL-2 Secretion by CTLA-4

(A) D2, D11, and D20 DC27.10-CTLA-4 TRIM KD cells (lanes 2–4, 6–8, 10–12, 14–16, 18–20) were compared with wild-type DC27.10-CTLA-4 cells (lanes 1, 5, 9, 13, 17) for the expression of TRIM (lanes 1–4), VAV-1 (lanes 5–8), SLP-76 (lanes 9–12), MAPK (lanes 13–16), and actin (lanes 17–20) by immunoblotting.

(B) DC27.10-CTLA-4 TRIM KD and WT cells were stained for CTLA-4 and CD3 surface expression. FACS profiles and percentage of CTLA-4 and CD3 surface expression in DC27.10-CTLA-4 TRIM KD cells relative to WT cells are shown.

(C) DC27.10-CTLA-4 TRIM KD and WT cells were stimulated with plate-bound anti-CD3 and anti-CD3 and CTLA-4 for 24 hr (anti-CD3 1 μg/ml, anti-CTLA-4 10 μg/ml). Supernatants were analyzed for IL-2 by ELISA. Percentage of inhibition in anti-CD3 and anti-CTLA-4-treated cells was calculated in comparison to anti-CD3-stimulated cells. Bar graphs show mean ± SD. Similar results were obtained from at least three other independent experiments.

throughout the stimulation period. The increase in fluorescence intensity was more pronounced when compared to mock transfected cells (Figure 3A). Total cell-associated PE fluorescence was then compared to the amount of internalized PE-labeled CTLA-4. This was determined by treatment with acidified PBS to remove surface-bound antibody, as described previously (Cefai et al., 1998). Consistent with increased CTLA-4 expression, TRIM-transfected cells also showed an increase in internalized CTLA-4. The amount of internalized CTLA-4 increased with TRIM expression (Figure 3A). However, the net effect was a higher sCTLA-4 expression over the time course (Figure 3B). This contrasted with TCR-CD3 expression, previously reported to bind to TRIM, that was not affected by increased TRIM expression and ionomycin treatment (Figure 3C; Bruyns et al., 1998; Kirchgessner et al., 2001). Taken together, our findings indicate that TRIM increased CTLA-4 surface expression, leading to increased internalization. However, internalization failed to fully remove the newly expressed sCTLA-4, allowing for the retention of increased CTLA-4 on the cell surface.

Upregulation of CTLA-4 by TRIM suggested that TRIM might indirectly alter T cell function. To test this, DC27.10-CTLA-4 cells were transfected with HA-TRIM and either left unstimulated or activated with anti-CD3, anti-CTLA-4, or anti-CD3 and anti-CTLA-4. After 24 hr, supernatants were taken and assessed for IL-2

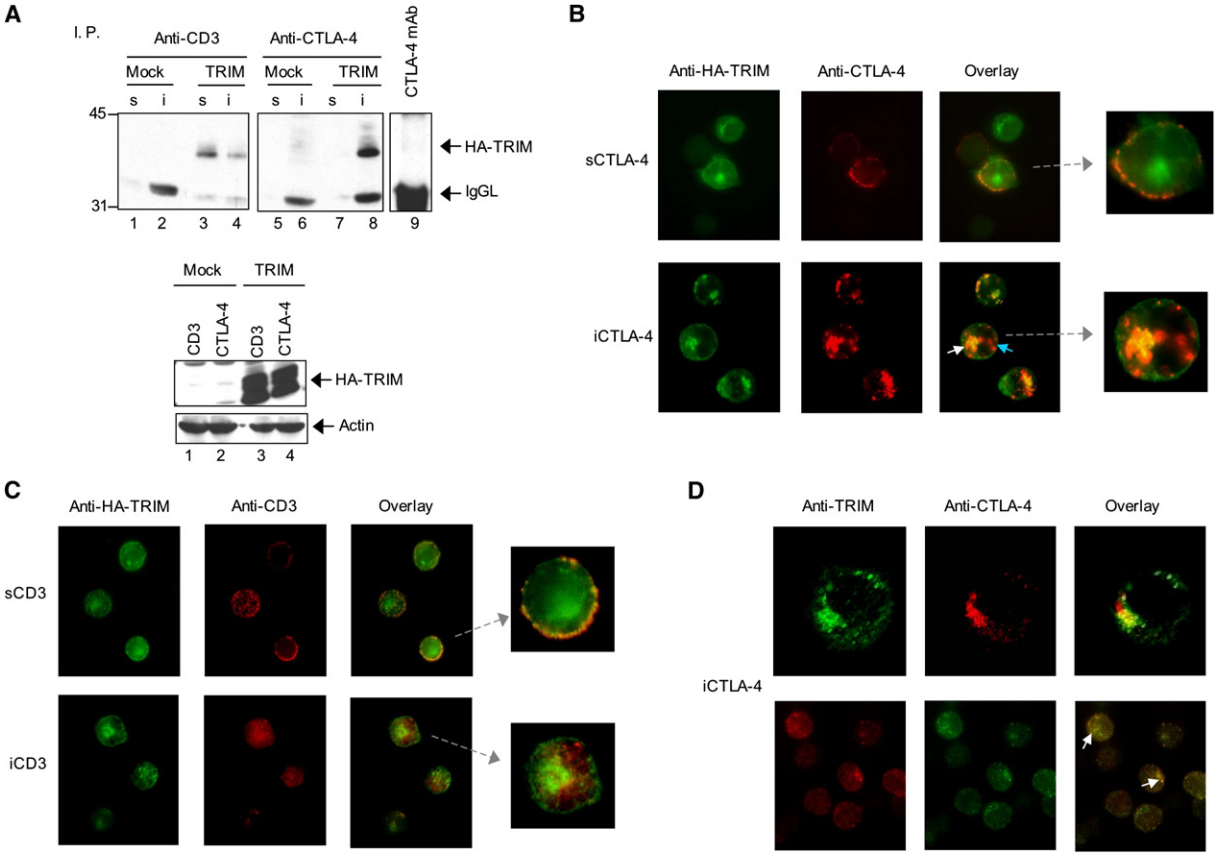


Figure 5. TRIM and CTLA-4 Colocalization and Binding Occurs Mainly Intracellular

(A) Surface CD3 and CTLA-4 from mock (lanes 1, 5) or HA-TRIM (lanes 3, 7)-transfected DC27.10-CTLA-4 cells were precipitated by preincubating intact cells with anti-CD3 (lanes 1–4) or anti-CTLA-4 (lanes 5–8) at 4°C, followed by washing and immunoprecipitation. Lysates were precleared with Protein A-Sepharose, followed by a second precipitation to purify intracellular CD3 (lanes 2, 4) or CTLA-4 (lanes 6, 8). Precipitates (lanes 1–8) and anti-CTLA-4 only (lane 9) were blotted with anti-HA. Expression of HA-TRIM and loading controls are shown in cell lysates immunoblotted with anti-HA and anti-actin, respectively.

(B and C) DC27.10-CTLA-4 cells were transfected with HA-TRIM, fixed in 4% PFA, and permeabilized with saponin. Cells were stained with anti-CTLA-4 and anti-mouse-Texas red (B) or anti-CD3 and anti-hamster-Texas red (C) either before fixation (surface) or after permeabilization (intracellular) and anti-HA-FITC to detect HA-tagged TRIM.

(D) Activated human PBLs were fixed, permeabilized, and stained for endogenous TRIM with anti-TRIM and anti-mouse Texas red and CTLA-4 with biotinylated anti-CTLA-4 and Streptavidin-FITC and analyzed by confocal microscopy. The results are representative of at least three experiments.

production (Figure 3D). Anti-CD3 and CTLA-4-mediated IL-2 production was markedly reduced in TRIM-transfected cells when compared to mock transfectants (65% versus 35%). Anti-CTLA-4 titration experiments revealed that the difference between TRIM- and mock transfected cells was most substantial with suboptimal concentration of anti-CTLA-4 (i.e., 7% inhibition in mock transfected cells compared to 56% inhibition in TRIM transfectants). Similarly, PBLs transiently transfected with HA-TRIM or mock were stimulated with anti-CD3, anti-CTLA-4, or anti-CD3 and CTLA-4. Proliferation of cells was then assessed after 36 hr. A subsequent increase in the inhibitory effect of CTLA-4 was observed on proliferation in TRIM-transfected primary T cells (Figure 3D). Compared to mock transfected cells, increased TRIM expression enhanced CTLA-4-mediated inhibition of proliferation by an additional 34% (13% versus 47%). In summary, these data demonstrate that TRIM enhancement of CTLA-4 surface expression resulted in a marked increase in CTLA-4-mediated inhibition of T cell function.

Downregulation of TRIM by shRNA Reduces CTLA-4 Surface Expression and Inhibition of IL-2 Production

Having demonstrated that TRIM overexpression leads to an upregulation of CTLA-4 surface expression, we used a complementary approach with shRNA to downregulate TRIM expression (Figure 4). Different DC27.10-CTLA-4 TRIM knockdown (KD) clones were identified with greatly reduced TRIM expression as shown by immunoblotting (Figure 4A). At the same time, the expression of other proteins such as VAV-1, SLP-76, MAPK, and actin was unaffected. Thus, shRNA TRIM resulted in the specific reduction of TRIM expression.

The reduction of TRIM expression led to reduced expression of sCTLA-4 when compared to DC27.10-CTLA-4 WT cells (Figure 4B). TRIM KD cells showed 60% less sCTLA-4 than WT cells. The total CTLA-4 as detected by staining of permeabilized cells was unaffected (data not shown). Further, the expression of surface CD3 (sCD3) was not altered by TRIM (Figure 4B).

Inhibition of anti-CD3-induced IL-2 production by CTLA-4 was analyzed in DC27.10-CTLA-4 WT and

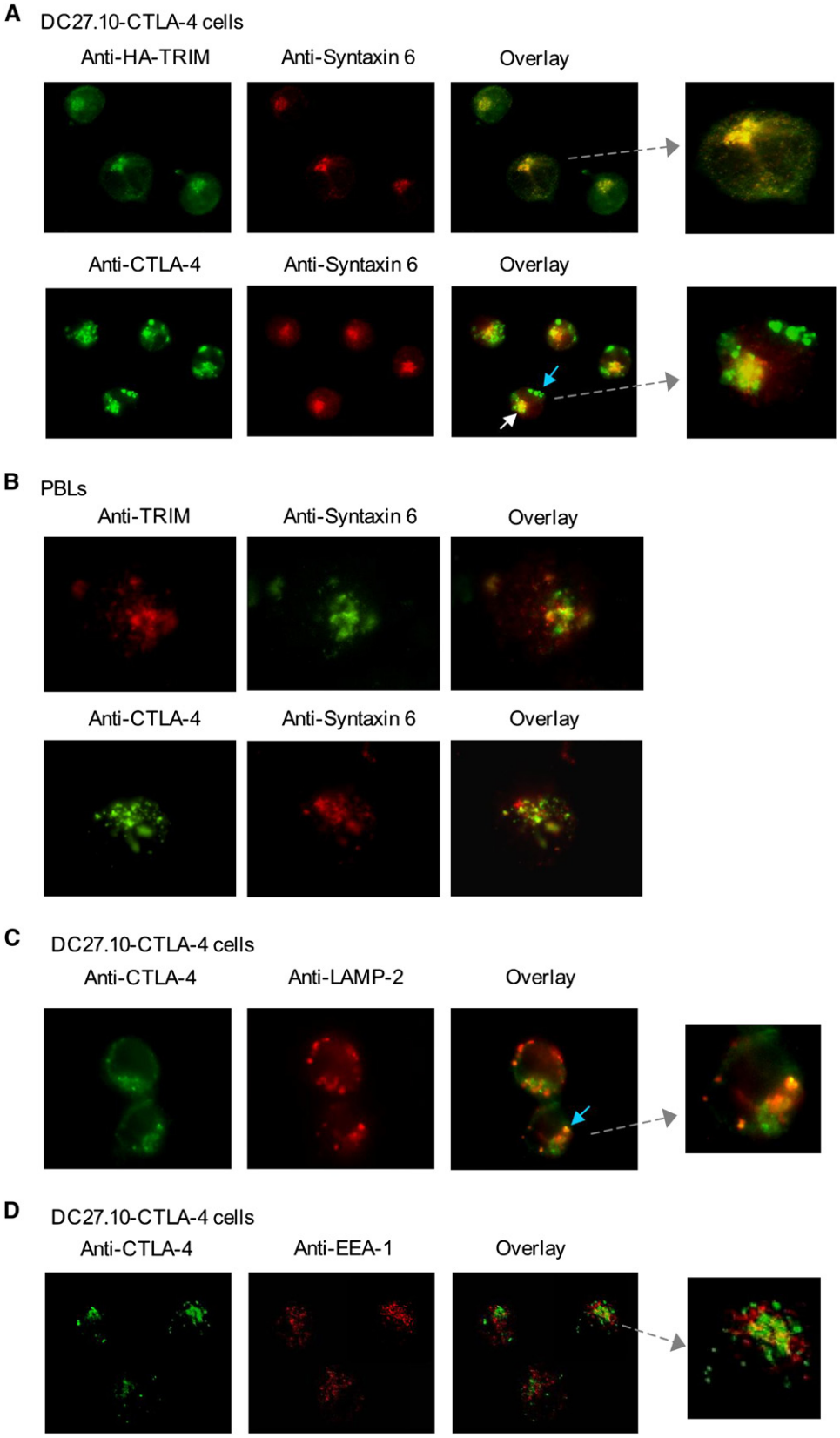


Figure 6. TRIM and CTLA-4 Colocalize Mainly in the TGN

(A) DC27.10-CTLA-4 cells were transfected with HA-TRIM, fixed in 4% PFA, and permeabilized with saponin. Cells were stained for HA-TRIM with anti-HA-FITC and Syntaxin 6 with anti-Syntaxin and anti-mouse Texas red or CTLA-4 with biotinylated anti-CTLA-4 and Streptavidin-FITC and Syntaxin 6 as indicated.

TRIM KD cells. WT and TRIM KD cells produced comparable amounts of IL-2 when stimulated with anti-CD3 (data not shown), whereas TRIM KD cells (D2, D11, D20) stimulated with anti-CD3 and anti-CTLA-4 showed impaired inhibition of IL-2 production when compared to WT cells (Figure 4C). Inhibition of IL-2 production in TRIM KD cells was 50%–60% less than that seen with WT cells. Overall, these findings confirmed a role for TRIM in upregulating CTLA-4 expression leading to an effect on CTLA-4 modulation of T cell function.

TRIM Colocalizes with CTLA-4 in Regions Distinct from the TCR-CD3 Complex

Because CTLA-4 is found mainly in intracellular compartments (Leung et al., 1995; Schneider et al., 1999), we next assessed the subcellular distribution of CTLA-4 and TRIM (Figure 5). Surface antigen was detected biochemically by preincubating cells with CTLA-4 mAb prior to washing, detergent solubilization, and immunoblotting as previously described (Schneider et al., 1999). Intracellular antigen was detected by depleting surface antigens followed by precipitation of the remaining intracellular material. sCD3 and sCTLA-4 as well as intracellular CD3 (iCD3) and iCTLA-4 were immunoprecipitated and blotted for HA-TRIM (Figure 5A). The majority of CTLA-4-associated TRIM was found intracellularly. Little if any material was detected on the cell surface. In contrast, anti-CD3 coprecipitated TRIM mainly from the cell surface with a small amount precipitated in intracellular compartments.

These biochemical results were confirmed by immunofluorescence staining. DC27.10-CTLA-4 cells were stained with FITC-conjugated anti-HA for detection of transfected HA-TRIM, whereas CTLA-4 was visualized with anti-CTLA-4 and Texas red-labeled secondary Ab. To distinguish between the surface and intracellular localization of CTLA-4, cells were stained either before or after fixation and permeabilization. As seen in Figure 5B, CTLA-4 and TRIM showed similar subcellular distribution patterns with extensive overlap (white arrow). Both were detected primarily intracellular with comparatively low expression at the plasma membrane. A small amount of CTLA-4 and TRIM colocalization occurred at the cell surface (top), whereas the majority of those complexes was found in intracellular compartments (bottom). This was in striking contrast with TRIM and the TCRζ-CD3 complex (Figure 5C; Bruyns et al., 1998; Kirchgessner et al., 2001). In this case, TRIM (FITC-labeled) and CD3 colocalization (Texas red) was detected almost exclusively at the cell surface (Figure 5C, top). These results confirmed the observation that CTLA-4 and TRIM initially bound in intracellular compartments. Further, it suggested that once transported to the surface, CTLA-4 and TRIM no longer exist as a complex.

The colocalization of CTLA-4 with endogenous TRIM was also confirmed in primary T cells (Figure 5D). PBLs were preactivated to induce CTLA-4 expression and subsequently stained for CTLA-4 and endogenous TRIM. As in the case of transfected HA-TRIM, colocalization with CTLA-4 occurred mainly in intracellular vesicles. Therefore, although TRIM associates with both TCR-CD3 and CTLA-4, there is a marked difference regarding the site of colocalization. CTLA-4 and TRIM complexes were detected mainly in intracellular compartments, whereas CD3 and TRIM associate at the plasma membrane.

TRIM and CTLA-4 Colocalize in the *trans* Golgi Network

Previous studies have reported the localization of CTLA-4 in several intracellular compartments including the *trans* Golgi network (TGN), lysosomes, and endosomes (Iida et al., 2000; Leung et al., 1995; Schneider et al., 1999). To assess in more detail where CTLA-4 and TRIM colocalize, cells were stained with anti-Syntaxin 6, a marker for the Golgi apparatus (Figures 6A and 6B; Shewan et al., 2003).

As shown in Figure 5B, CTLA-4 was found in a characteristic pattern of clusters of intracellular vesicles. Anti-TRIM staining overlapped with one set of these CTLA-4-stained vesicles. A similar pattern was observed for CTLA-4 and Syntaxin 6 costaining (see arrows Figure 6A, bottom). Because TRIM staining closely matched anti-Syntaxin 6 staining (Figure 6A, top), we concluded that intracellular TRIM and CTLA-4 association mainly occurred in the TGN. This could also be demonstrated in human PBLs (Figure 6B). Some CTLA-4-containing vesicles (see blue arrow) colocalized partially with early lysosomes as defined by anti-Lamp-2 staining (Figure 6C; Reuter et al., 2004). Only marginal overlap of CTLA-4 with early endosomal vesicles was observed as shown by anti-EEA1 (early endosome antigen 1) staining (Figure 6D; Christoforidis et al., 1999). Thus, the majority of TRIM and CTLA-4 colocalize in the TGN, the site from which newly synthesized CTLA-4 is released to the cell surface.

Modulation of the Localization Pattern of CTLA-4 by TRIM

To assess whether the localization of CTLA-4 was modulated by TRIM, DC27-10-CTLA-4–TRIM KD cells were stained for CTLA-4 and analyzed by confocal microscopy. As shown in Figures 6A–6D, the majority of CTLA-4 in wild-type cells was localized in the Golgi compartment and several clusters of vesicles, which partly costained with lyso- and endosomes. This localization pattern was altered in cells where TRIM expression was markedly reduced. Most of CTLA-4 in TRIM KD cells appeared to be centralized (Figure 7A, left) with fewer surrounding vesicles when compared to wild-type cells. Costaining for the Golgi marker Syntaxin

(B) PBLs were activated with PHA (2.5 μg/ml) for 4 days to induce CTLA-4 expression, fixed in 4% PFA, and permeabilized with saponin. Cells were stained for endogenous TRIM with goat anti-TRIM and anti-goat Texas red $F(ab)_2$ and Syntaxin 6 with anti-Syntaxin and anti-mouse FITC or CTLA-4 with biotinylated anti-CTLA-4 and Streptavidin-FITC and Syntaxin 6 with anti-Syntaxin and anti-mouse Texas red as indicated.
(C and D) DC27.10-CTLA-4 cells were transfected with HA-TRIM, fixed in 4% PFA, and permeabilized with saponin. Cells were stained for (C) CTLA-4 and Lamp-2 with anti-Lamp-2 and anti rat-Texas red; (D) CTLA-4 and EEA1 with anti-EEA1 and anti rabbit-Alexa Fluor 594 and analyzed by fluorescence microscopy (A, B, C) or confocal microscopy (D). Arrows indicate different sets of CTLA-4-stained vesicles (A, C). Results shown are representative of at least three experiments.

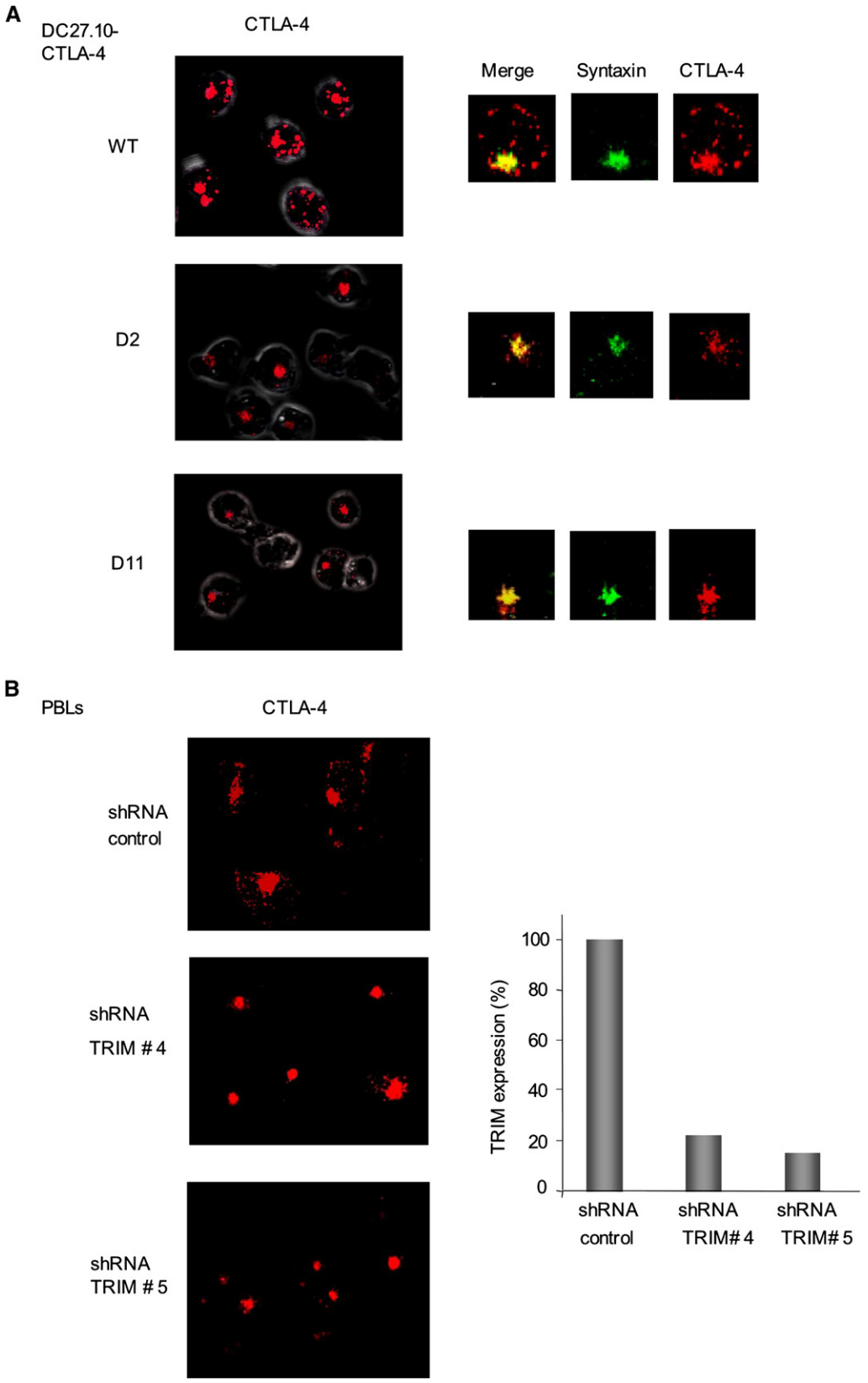


Figure 7. Subcellular Distribution of CTLA-4 Is Altered by TRIM shRNA Knockdown

(A) DC27.10-CTLA-4 TRIM KD and WT cells were fixed in 4% PFA and permeabilized with saponin. Cells were stained with anti-CTLA-4 and anti-mouse AlexaFluor 647 and analyzed by confocal microscopy (left). Double staining for CTLA-4 and Syntaxin 6 was performed with biotinylated anti-CTLA-4 and Streptavidin-Alexa Fluor 568 and anti-Syntaxin and anti-mouse Alexa Fluor 647 (right).

6 revealed that CTLA-4 was almost exclusively localized in the Golgi area in TRIM KD cells (Figure 7A, right).

The same change in the pattern of subcellular CTLA-4 localization could be observed in PBLs transiently transfected with TRIM shRNA when compared to PBLs transfected with control shRNA (Figure 7B, left). Although control cells showed CTLA-4 staining in distinct vesicles throughout the cell, detection of CTLA-4 was restricted to a centralized area in PBLs transfected with TRIM shRNA. TRIM expression in these cells was reduced by 80%–85% (Figure 7B, right). Overall, in TRIM KD cells as well as in primary cells transiently transfected with TRIM shRNA, there was a marked reduction of vesicles between the Golgi area and the plasma membrane, a result consistent with an impairment of transport from the TGN.

Discussion

Despite its potent effects on T cell function, CTLA-4 is primarily an intracellular antigen whose surface expression is tightly regulated (Alegre et al., 1996; Lindsten et al., 1993; Linsley et al., 1996). This regulation is achieved by restricted trafficking to the cell surface and rapid internalization. Even at its highest amounts, surface CTLA-4 is expressed at about 3% relative to CD28 (Lindsten et al., 1993). This tight regulation is presumably due to the need to control surface expression of this potent coreceptor because minor changes in surface expression could have major effects on the outcome of T cell activation. Intracellular CTLA-4 has been reported to be located in various compartments including the TGN, lysosomes, and endosomes (Alegre et al., 1996; Iida et al., 2000; Leung et al., 1995; Linsley et al., 1996). However, little is known regarding the regulation of intracellular trafficking pathways used by CTLA-4 to reach the plasma membrane. In this study, we demonstrate that TRIM facilitates the movement of CTLA-4 from the TGN to the surface of T cells. The adaptor was found to bind to CTLA-4 in intracellular compartments and to colocalize with CTLA-4 in the TGN. The amounts of CTLA-4–TRIM binding greatly exceeded that observed for TCR-CD3–TRIM. The interaction was specific in that it was not observed with CD28. Further, TRIM overexpression increased sCTLA-4 expression, whereas the reduction of TRIM expression by shRNA reduced sCTLA-4 expression and its ability to inhibit cytokine production. Furthermore, the reduction of TRIM changed the intracellular localization pattern of CTLA-4, with the coreceptor being centralized and restricted to the Golgi compartment. There was a striking reduction of vesicles between the Golgi area and the plasma membrane, which are presumably transport vesicles. Overall, our findings identified a role for TRIM as a chaperone that affects T cell function by modulating CTLA-4 cell-surface expression.

CTLA-4 surface expression is a highly dynamic process, being recycled between intracellular stores and the cell surface. Upon T cell activation, CTLA-4 is released toward the immunological synapse, the site of TcR-CD3–APC engagement (Linsley et al., 1996). Punctual and directed CTLA-4 surface expression is essential for its function at the immunological synapse and modulation of T cell activation. Previous studies have shown that CTLA-4 externalization can be augmented by increased intracellular Ca^{2+} concentrations and by mutation of the YVKM motif (Linsley et al., 1996; Yi et al., 2004). Mutation of the tyrosine in this motif will enhance CTLA-4 surface expression (Yi et al., 2004). As in the case of many receptors, phospholipase D (PLD) and small ADP ribosylation factor (ARF) are required for transport of CTLA-4 from the TGN to the cell surface (Mead et al., 2005). Presumably, other key regulators of trafficking (i.e., SNAREs) are also needed for CTLA-4 expression. TRIM differs from these mediators in specifically binding to CTLA-4. This immune-specific interaction presumably operates in conjunction with more general constituents involved in the trafficking process. The manner by which this complex interacts with generic events remains to be determined.

The ability of TRIM to increase cell-surface expression was demonstrated by pulse-chase and FACS analysis. Pulse-labeled CTLA-4 appeared more quickly and at higher amounts on the surface of T cells in TRIM-transfected cells, whereas FACS analysis showed TRIM-mediated increased CTLA-4 surface expression leading to increased internalization. However, increased internalization failed to fully remove the newly expressed sCTLA-4, resulting in retention of increased CTLA-4 on the cell surface. In this regard, increased internalization is most likely a reflection of the capacity of the endocytic process to accommodate newly expressed sCTLA-4. It is unlikely to reflect an actual increase in the role of internalization per se. Overall, this finding suggests that not only does TRIM associate with CTLA-4 and colocalize in the TGN, but serves as a shuttle protein to facilitate CTLA-4 surface expression. Independent of TRIM binding, CTLA-4 is translocated to endo- and lysosomes, and either degraded or recycled to the cell surface. Rapid endocytosis of CTLA-4 is facilitated by AP-2 (Bradshaw et al., 1997; Chuang et al., 1997; Shiratori et al., 1997; Zhang and Allison, 1997), whereas AP-1 regulates trafficking from the Golgi to lysosomal compartments (Schneider et al., 1999). The binding of TRIM to CTLA-4 occurs independently of AP-1 and AP-2 binding. Mutation of the Y201 residue in CTLA-4 cytoplasmic tail abrogates AP-1–AP-2 binding (Schneider et al., 1999), whereas binding to TRIM was not influenced.

The properties associated with TRIM and CTLA-4 binding are distinct from those linked to TRIM and TCR-CD3. Although TRIM–CTLA-4 binding occurs mainly inside the cells leading to increased surface expression, the binding of TRIM to TCR-CD3 occurs primarily at the cell surface. Little if any TRIM was found associated with CTLA-4 on the cell surface. This finding is in agreement with a model in which TRIM facilitates CTLA-4 surface expression followed by TRIM dissociation from CTLA-4 and binding of TRIM to the TCR-CD3

(B) PBLs were transfected with TRIM shRNA and stimulated with PHA (2.5 μg/ml) for 3 days. Cells were fixed in 4% PFA, permeabilized with saponin, and stained for CTLA-4 with biotinylated anti-CTLA-4 and Streptavidin-Alexa Fluor 568. Mock and shRNA-TRIM-transfected cells were analyzed by confocal microscopy (left). TRIM expression in PBLs and shRNA TRIM-transfected cells was assessed by immunoblotting (data not shown) and densitometric analysis (right). Results are representative of at least two experiments.

complex. TRIM overexpression did not alter the expression of the TCR-CD3 complex in a hybridoma and primary T cells. Further, the function of TRIM mediated release of CTLA-4 to the cell surface is in contrast to the mechanism described for enhanced TCR-CD3 expression mediated by the association with TRIM. The interaction of TRIM with TCR-CD3 leads to reduced internalization of the TcR-CD3 complex (Kirchgessner et al., 2001), whereas the interaction of TRIM with CTLA-4 leads to increased externalization of CTLA-4 in DC27.10-CTLA-4 cells as well as in primary T cells. The differences in the proposed mechanisms of TRIM-mediated enhanced surface expression of CD3 and CTLA-4, respectively, is reflected in the main sites of colocalization. While TcR-CD3–TRIM interaction has been observed mainly at the cell surface, CTLA-4–TRIM association occurs predominately in the TGN. Increased TcR-CD3 expression mediated by TRIM overexpression is reported for the Jurkat T cell line (Kirchgessner et al., 2001). It is possible that this cell line has its own property in terms of reduced internalization of the TCR-CD3 complex after TRIM transfection. Also, other studies on the Jurkat T cell line found that there is no requirement for CD28-PI3-kinase binding in anti-CD3-induced IL-2 production. This contrasts with results obtained from other cell lines and is likely due to the loss of the phosphatase and tensin homolog PTEN in Jurkat cells (Shan et al., 2000). During the preparation of this manuscript, Kölsch et al. reported that in TRIM-deficient mice there is no alteration in TCR-CD3 expression when compared to wild-type mice (Kölsch et al., 2006).

TRIM expression was found to potentiate surface expression of CTLA-4 with the net effect to enhance indirectly the suppression of T cell activation (i.e., IL-2 production, proliferation). CTLA-4 is present in enlarged, abnormal vesicles in Chediak Higashi syndrome (CHS), and T cells from patients suffering from CHS were shown to be defective in cycling CTLA-4 to the cell surface after stimulation. Therefore, defective surface expression of CTLA-4 by CHS T cells is involved in the generation of lymphoproliferative disease (Barrat et al., 1999). Our data show that TRIM modulates T cell responses by affecting CTLA-4 surface expression. Although the effect of TRIM on CTLA-4 function is due to an increase in CTLA-4 surface expression, further functions for TRIM cannot be excluded. TRIM might additionally enhance CTLA-4 signaling directly as a typical adaptor protein by recruiting downstream signaling elements (i.e., PI3-kinase, src-kinases) (Bruyns et al., 1998). It is unlikely that TRIM itself can operate to facilitate a negative immune response, independently of CTLA-4, given that the adaptor failed to modulate T cell responses on its own (Bruyns et al., 1998; Kirchgessner et al., 2001; Kölsch et al., 2006). The mild phenotype of the TRIM-deficient mice suggests that other nonraft TRAPs may compensate for the absence of TRIM (Kölsch et al., 2006). Other TRAPs may eventually be found to associate with CTLA-4. For example, LAX-deficient T and B lymphocytes are hyperresponsive to anti-CD3 and anti-IgM-mediated stimulation (Zhu et al., 2005), and SIT-deficient mice show an increased susceptibility to develop experimental autoimmune encephalomyelitis (Simeoni et al., 2005). It is not surprising that TRIM-deficient mice failed to show an autoimmune phenotype because the knockdown of TRIM in our study did not lead to a complete loss of surface CTLA-4. This result suggests that there are other proteins involved in the regulation of CTLA-4 expression.

Our findings demonstrating that TRIM facilitates CTLA-4 surface expression followed by TRIM dissociation and association with the TCR-CD3 complex on the cell surface suggest that TRIM may promote CD3–CTLA-4 localization at the immunological synapse, which has been shown to be a crucial event in CTLA-4-mediated inhibition of T cell activation (Darlington et al., 2002; Egen and Allison, 2002). An intriguing aspect of TRIM is its ability to associate with both of these two central receptors in the regulation of T cell activation. The functional relevance of interconnecting the TCR-CD3 complex and CTLA-4 remains to be elucidated.

Experimental Procedures

Cells and Reagents

DC27.10-CTLA-4 cells were cultured in RPMI media supplemented with fetal calf serum as described elsewhere (Cai et al., 1995; Schneider et al., 1999). Human peripheral T cells were isolated from buffy-coats from healthy individuals (Colingdale and Addenbrooke's NHS Blood Donor Service, London and Cambridge, UK) as described (Schneider et al., 1999). Anti-human CD3 (OKT3) and anti-SLP-76 were obtained from American Type Culture Collection. Anti-human CTLA-4 (BNI3) was kindly provided by B. Broeker (Greifswald, Germany). Anti-mouse CD3 (145-2C11), anti-mouse CTLA-4 (UC10-4F10-11), and anti-mouse CD28 (37.51) were purchased from BD Pharmingen.

Antibodies and reagents used for immunofluorescence staining and biochemistry were purchased as follows: anti-TRIM (TRIM-04) and anti-EEA1 from abcam; anti-human CTLA-4-biotin, anti-human-CD3-FITC, anti-Syntaxin 6, and Streptavidin-FITC from BD Biosciences; anti-Lamp2 (ABL-93) from Southern Biotech; anti-mouse-Texas Red and anti-mouse-FITC from Jackson ImmunoResearch; anti-HA-FITC from Covance; anti-human CTLA-4-PE and mouse IgG2a-PE from Beckman Coulter; and anti-rabbit-Alexa Fluor 594, anti-mouse Alexa Fluor 647, and Streptavidin-Alexa Fluor 568 from Molecular Probes. Anti-VAV and anti-MAPK antibodies were from New England Biolabs.

Plasmid Construction

Full-length human TRIM cDNA was isolated via RT-PCR. Total RNA was isolated from anti-CD3-stimulated Jurkat cells with the Trizol reagent (Invitrogen). PCR primer sequences were sense, 5-ccgaaaa aaagaagcatgtcagg-3 and antisense, 5-catggtccagctagtttataggttc-3. The amplified products were ligated into the TA cloning vector (Invitrogen) and confirmed by DNA sequencing. An expression vector encoding the influenza virus hemagglutinin (HA)-tagged TRIM was constructed by cloning the 560 bp SacI-KpnI fragment of TRIM into the SacI-KpnI site of the expression vector pSRα in-frame with the HA-tag at the C terminus.

The template for mouse TRIM shRNA were generated by ligating the annealed primers 5′-acctcggatgagaactgctatgaacatcaagagtgttc atagcagttctcatcctt-3′ and 5′-caaaaaggatgagaactgctatgaacactcttga tgttcatagcagttctcatccg-3′ and 5′-acctcgctatgcctcactggatcacatcaa gagtgtgatccagtgaggcatagctt-3′ and 5′-caaaaagctatgcctcactggatca cactcttgatgtgatccagtgaggcatagcg-3′ into the BbsI sites of psiRNA-hH1GFPzeo G2 vector (InvivoGen). As a control, the primers 5′-ac ctcgcgttaattagactgaggagttcaagagactcctcagtctaattaacgctt-3′ and 5′-caaaaagcgttaattagactgaggagtctcttgaactcctcagtctaattaacggg-3′ were used. Designed RNA oligonucleotides were blasted against the GenBank/EMBL/DDBJ database to ensure gene specificity.

The construction of the CTLA-4 plasmids (wild-type and mutants) is described (Schneider et al., 1999).

Transfections

DC27.10-CTLA-4 cells or PBLs were transiently transfected by a standard electroporation protocol (Schneider et al., 1999) or by

the Amaxa Nucleofector Kit, respectively. 18 hr (for PBLs) and 24 hr (for DC27.10-CTLA-4 cells) after transfection, cells were stimulated as described below.

Interleukin-2 Assay

DC27.10-CTLA-4 and DC27.10-CTLA-4 TRIM KD cells (1×10^5/200 μl) were stimulated with plate-bound anti-CD3 (1 μg/ml) and anti-CTLA-4 (10 μg/ml, 20 μg/ml). After 24 hr, supernatants were taken and IL-2 concentration determined by ELISA (BD Biosciences) according to manufacturer's protocol.

Proliferation Assay

Purified naive T cells were cultured at a density of 2×10^5/200 μl cells in the presence of mitomycin C-treated autologous APCs (ratio 5:1) in anti-CD3 (2 μg/ml) and anti-CTLA-4 (10 μg/ml)-coated 96-well plates for 36 hr. To assess proliferation, cells were pulsed with 1 μCi of [^{3}H]thymidine for the last 12 hr of the indicated period of time.

Flow Cytometry and Immunofluorescence

To analyze CTLA-4 expression, cells were stained for surface CTLA-4 with CTLA-4-PE mAb or PE-labeled isotype control antibody. Alternatively, CTLA-4 was labeled with anti-CTLA-4 and anti-mouse-APC secondary antibody and only secondary antibody as control. For intracellular HA-TRIM staining, cells were fixed in 4% paraformaldehyde (PFA) (Sigma), permeabilized with 0.3% saponin (Sigma), and stained with anti-HA-FITC in saponin containing PBS/BSA. Cells were fixed and analyzed with a FACSCalibur flow cytometer.

For immunofluorescence staining, cells were fixed in 4% PFA, permeabilized with 0.3% saponin, and stained with anti-CD3, anti-CTLA-4, anti-Syntaxin, anti-TRIM, and appropriate fluorochrome-labeled secondary antibody or anti-HA-FITC, respectively. Additionally, biotinylated anti-CTLA-4 and fluorochrome-labeled Streptavidin were used. Negative controls were run with unstained, single-stained samples, or omitting primary antibody. Stained cells were mounted on slides and analyzed by fluorescence microscopy.

Ionomycin-Induced CTLA-4 Surface Expression

Isolated human PBLs were stimulated with 1 μM ionomycin (Sigma) for up to 3 hr at 37°C. Samples were stored on ice and subsequently stained for CTLA-4 and HA-TRIM expression as indicated. To determine exocytosis of CTLA-4 over time, PBLs were labeled with anti-CTLA-4-PE in AIMV medium plus 2% FCS for 30 min on ice and subsequently activated with ionomycin at 37°C in the presence of anti-CTLA-4-PE (1 μg/ml) for up to 3 hr. At the indicated times, cells were washed once with PBS and fixed in 2% PFA, and total cell-associated fluorescence was analyzed by flow cytometry.

For comparison of total cell-associated fluorescence (surface and internalized CTLA-4) and intracellular PE fluorescence (internalized CTLA-4), samples were divided in two parts. One part was left untreated, while the other was incubated on ice for 45 s in PBS acidified to pH 2.0 and supplemented with 0.03 M sucrose and 10% FCS (Cefai et al., 1998) to remove surface-bound antibody. Cells were washed and analyzed by FACS. Untreated samples account for total cell-associated fluorescence, while acid-stripped aliquots account for PE fluorescence in acid-resistant (internal) compartments.

Immunoprecipitation and Immunoblotting

For immunoprecipitation, cells were lysed in ice-cold lysis buffer containing 1% TritonX-100 in 20 mM Tris-HCl (pH 8.3), 150 mM NaCl. The lysis buffer contained protease and phosphatase inhibitors. Postnuclear lysates were incubated for 1 hr with the indicated antibody. Protein A-Sepharose beads (30 μl, Amersham Pharmacia) were added and incubated for 1 hr at 4°C. The eluted proteins (nonreducing conditions for CD28 and detection of endogenous TRIM) were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting. The membranes were blocked with 5% milk in TBS (10 mM Tris-HCl [pH 7.6], 150 mM NaCl) and incubated with the indicated antibody. Bound antibody was revealed with the appropriate secondary antibody, and protein was visualized by enhanced chemiluminescence (ECL, Amersham).

Metabolic Labeling

To monitor CTLA-4 surface expression of newly synthesized CTLA-4 in mock or TRIM-transfected DC27.10-CTLA-4 cells, cells were pulsed with [^{35}S]methionine for 20 min and chased with nonradioactive methionine for the indicated periods of time in the presence of ionomycin (1 μM). Surface CTLA-4 from both transfectants was precipitated by preincubating labeled cells with anti-CTLA-4 at 4°C, followed by washing and immunoprecipitation. Lysates were subjected to preclearing with Protein A-Sepharose, followed by a second precipitation to purify intracellular CTLA-4. The immunoprecipitates were analyzed by 10% SDS-PAGE.

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