CTL-Associated Antigen-4 Ligation Induces Rapid T Cell Polarization That Depends on Phosphatidylinositol 3-Kinase, Vav-1, Cdc42, and Myosin Light Chain Kinase

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CTL-Associated Antigen-4 Ligation Induces Rapid T Cell Polarization That Depends on Phosphatidylinositol 3-Kinase, Vav-1, Cdc42, and Myosin Light Chain Kinase

Bin Wei,* Silvy da Rocha Dias,* Hongyan Wang,* and Christopher E. Rudd2*†

CTLA-4 can negatively regulate cytokine production and proliferation, increase motility, and override the TCR-induced stop-signal needed for stable T cell-APC conjugation. Despite this, little is known regarding whether CTLA-4 can alter T cell morphology and the nature of the signaling events that could account for this event. In this study, we demonstrate that anti-CTLA-4 and CD3/CTLA-4 induce rapid T cell polarization (i.e., within 15–30 min) with increases in lamellipodia, filopodia, and uropod formation. This was observed with anti-CTLA-4 and CD80-Ig ligation of CTLA-4, but not with anti-CD3 alone, or anti-CD3/CD28 coligation. Polarization required PI3K, the guanine nucleotide exchange factor Vav1, the GTP-binding protein Cdc42, as well as myosin L chain kinase. By contrast, a key downstream target of PI3K, protein kinase B, as well as Rho kinase and RhoA, were not needed. Our results demonstrate that CTLA-4 is a potent activator T cell polarization needed for motility, and this process involves specific set of signaling proteins that might contribute to coreceptor regulation of T cell function. The Journal of Immunology, 2007, 179: 400–408.

The T cell response depends on a convergence of signals from the AgR (TCR/CD3) and coreceptors such as CD28, ICOS, and CTLA-4 (1–3). The inhibitory property of CTLA-4 on cytokine production and proliferation has been observed in Ab coligation experiments (4) and by the phenotype of the CTLA-4-deficient mice (1, 5). CTLA-4+/− mice die at an age of 2–3 wk due to an autoimmune pathology with massive lymphocytic infiltration of major organs. Modulation of CTLA-4 can enhance autoimmunity and antitumor responses and lowers the threshold needed for response to Ag (1, 6).

CTLA-4 is related to CD28 in binding to ligands CD80/86 and in binding to PI3K and up-regulating JNK activation (7, 8). Negative signaling has been reported due to binding to phosphatases protein phosphatase 2A or Src homology region 2 domain-containing phosphatase-2 (9–11). Similarly, the coreceptor can inhibit the activation of ERKs and enter/interfere with lipid raft expression (8, 11, 12–15). Clathrin-associated adaptor protein complexes AP-1 and AP-2 bind to CTLA-4 (16–18). Although AP-2 facilitates CTLA-4 endocytosis, AP-1 promotes transport to lysosomes (16–19). Src kinase-mediated phosphorylation can also modulate endocytosis of the coreceptor (20–22).

Recently, we showed that CTLA-4 ligation regulates the activation of integrins and can influence TCR complex up-regulation (i.e., inside-out signaling) of LFA-1 adhesion (23). Paradoxically, reduced adhesion of CTLA-4+/− T cells occurs concurrently with enhanced proliferation and IL-2 production (1–6, 23). Anti-CTLA-4 can activate the integrin regulator Rap1 (23, 24), increase motility, and reverse the TCR-induced stop-signal needed for stable T cell-APC interactions (25). In this context, T cell activation is accompanied by altered morphology with cell polarization (26–32). Polarization and motility require contractile actin-myosin and lamellipodia formation (26, 27). Actin in lamellipodia forms a branching network, whereas parallel bundles form in filopodia (28). Reorganization of the cytoskeleton is needed for T cell activation and function (29–32).

Rac and Cdc42 regulate lamellipodial and filopodial protrusions, respectively, whereas Rho, Rac, and Cdc42 promote integrin-based adhesion complexes (33–36). PI3K-derived phosphatidylinositol 3,4,5-trisphosphate (PIP3)3 is also needed for polarization, whereas the Rho family modulates actin-myosin filaments with mDia and p160ROCK (27, 28, 34–36). Cdc42 activation has been implicated in phosphatase and tensin homolog deleted on chromosome 10 exclusion from protrusions in leukocytes (37). The guanine exchange factor Vav-1 also mediates actin polymerization and, consequently, TCR clustering and cell spreading (38–41). Furthermore, LFA-1 binding to ICAM-1 can induce polarization via myosin L chain kinase (MLCK)-mediated attachment and Rho kinase (ROCK)-dependent detachment (42).

Given the ability of CTLA-4 ligation to regulate LFA-1-mediated adhesion and motility (23, 24), it was of interest to assess coreceptor signaling on T cell morphology. In this study, we demonstrate that CTLA-4 induces an unusually rapid polarization of T cells mediated by a specific set of mediators that include PI3K, Vav1, Cdc42, and MLCK.

1 Abbreviations used in this paper: PIP3, phosphatidylinositol 3,4,5-trisphosphate; GEF, guanine nucleotide exchange factor; GSK-3, glycogen synthase kinase-3; HA, hemagglutinin; MLCK, myosin L chain kinase; PH, pleckstrin homology; PKB, protein kinase B; ROCK, Rho kinase.

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Materials and Methods

Abs, plasmids, and inhibitors

Anti-mouse CD3 (145-2C11), anti-human CD3 (OKT3), and anti-hemagglutinin (HA) (12CA5) were obtained from American Type Culture Collection, anti-human CTLA-4 (BNI3), anti-human CD28 (9.3), and anti-CTLA-4 mAbs were obtained from Dr. B. Broker (Ernst-Moritz-Arndt University, Greifswald, Germany). Phalloidin-FITC and phalloidin-tetramethylrhodamine isothiocyanate was purchased from Sigma-Aldrich (P5282 and P1951). Anti-myc and anti-Vav1 was purchased from Upstate Biotechnology. Anti-phospho-AKT1/2 was purchased from BD Pharminogen. Myc-tagged Vav1L213A (Vav1GEF*) and Vav1PH were gifts from M. Villalba (Institut Federatif de Recherche, Montpellier, France). Recombinant CD80Ig was purchased from R&D Systems. The inhibitors used were LY294002, toxin A clostridium difficile, ML-7, C3 enzyme, SH-5, and Y27632 bought from Calbiochem.

Cell culture and cell sorting

Murine T cell hybridoma DC27.10-CTLA-4-expressing CTLA-4, DC27.10-CTLA-4 Y201F-expressing CTLA-4 Y201F mutant, and DC27.10-CD28-expressing CD28 were cultured in complete RPMI 1640 medium and supplemented with 5% FCS and 50 μM 2-ME (23). Human peripheral blood lymphocytes cells were prepared from healthy volunteers by standard Ficoll-Hypaque centrifugation and cultured in the presence of plate-bound anti-CD3 plus anti-CD28 for 48 h, and CTLA-4-positive T cells were sorted using a FACSVantage (BD Biosciences).

Cell polarization induction

Cells were plated on plates with immobilized anti-CD3 (2 μg/ml), anti-CD28 (5–40 μg/ml), and/or anti-CTLA-4 (5–40 μg/ml). Anti-CTLA-4 and CD28 Abs were added at designated concentrations (5–40 μg/ml) in a 96-well microtiter plates. DC27.10-CTLA-4, DC27.10-CD28, or primary T cells were plated at a density of 1.5 × 10^4 cells/well and incubation for 15 min to 6 h at 37°C. All the imaged were acquired using an inverted Nikon Diaphot-300 fluorescence microscope.

Transfection and immunoblotting

Cells in a logarithmic growth phase were transfected with the indicated amounts of plasmid DNAs by electroporation as described previously (43). In each experiment, cells were transfected with plasmid DNA by supplementing expression vector DNA with the proper amounts of the corresponding empty vector. Cell morphology analysis was performed 24 h after transfection as described for each experiment. Immunoblotting were performed as described previously (43).
Results

CTLA-4 induces rapid T cell polarization

We recently showed that anti-CTLA-4 up-regulates LFA-1-mediated adhesion (23). To investigate whether anti-CTLA-4 can alter T cell morphology, CTLA-4-expressing DC27.10 hybridoma T cells (DC27.10-CTLA-4) were ligated with Abs and stained with FITC-phalloidin. Anti-CD3/CTLA-4 induced an elongated polarized morphology over 4 h that was not observed with anti-CD3 or anti-CD3/CD28 or with anti-CD3/CTLA-4 ligation of CTLA-4-negative DC27.10 cells. Left vertical panels, DC27.10 cells; right vertical panels, DC27.10 CTLA-4 cells. B, Numbers of cells with lamellipodia, filopodia, and uropods following anti-CD3, anti-CD3/CD28, or anti-CD3/CTLA-4. Upper panels, Examples of images of cells with filapodia (a), filopodia/ lamellipodia (b), lamellipodia (c), and uropod (d). Lower panels, Histograms showing the numbers of cells lamellipodia, filopodia, and uropods. a, Unstimulated; b, anti-CD3; c, anti-CD3/CD28; and d, anti-CD3/CTLA-4. C, Numbers of primary T cells with lamellipodia, filopodia and uropods following anti-CD3, anti-CD3/CD28 or anti-CD3/CTLA-4. a, Unstimulated; b, anti-CD3; c, anti-CD3/CD28; and d, anti-CD3/CTLA-4.

Although slight changes in morphology were occasionally observed with anti-CD3 and anti-CD3/CD28 over this time course, this was seen at the level far below that for anti-CD3/CTLA-4 (i.e., 10–15% for anti-CD3 vs 60–70% for anti-CD3/CTLA-4). This occurred without a loss of cell viability as determined by trypan blue exclusion (data not shown). To exclude the possibility that the anti-CD3/CTLA-4 effects were simply due to an increase in the Ab concentration on plates, an Ab titration experiment was performed (Fig. 1B). Anti-CD3 at 2 μg/ml was combined with anti-CTLA-4 at 5, 10, 20, and 40 μg/ml to ligate DC27.10-CTLA-4 cells (left histogram). A parallel titration was performed using anti-CD3 with the same increase in concentration of anti-CD28 with DC27.10-CD28 cells (right histogram). Although the titration of anti-CTLA-4 increased levels of polarization, no effect was observed with anti-CD28. This indicated that the polarization was concentration-dependent and specific for CTLA-4.

The same cells were treated with immobilized anti-CD3/CD80 Ig for 4 h, and CD80-Ig was also found effective in inducing...
polarization. In one example, >40–70% DC27.10-CTLA-4 cells ligated with anti-CD3/CD80-Ig and 25% ligated with CD80-Ig alone showed an elongated phenotype (Fig. 1C). The level of spreading increased with increasing amounts of CD80-Ig (i.e., 2 vs 4 μg/ml). By contrast, anti-CD3/CD80-Ig had no effect on DC27.10 cells or DC27.10 cells transfected with the other ligand CD28. Lastly, pre-activated and sorted CTLA-4-positive primary human peripheral T cells (>90% CTLA-4 positive) were also ligated with Abs (Fig. 1D; see upper panels). Over 35% of anti-CD3/CTLA-4-ligated cells became elongated, compared with 22% with anti-CTLA-4 alone and 5–8% of untreated or anti-CD3- or anti-CD3/CD28-treated cells (lower histogram). Overall, these observations indicate that CTLA-4 coligation with CD3 has relatively profound effect on T cell polarization (i.e., 1–3 h) that was not observed with CD28 coligation. These observations indicate that CTLA-4 ligation by Ab or CD80-Ig ligand can potently and rapidly induce T cell polarization.

**Anti-CTLA-4 ligation induces increased lamellipodia, filopodia, and uropod formation**

To examine these morphological effects in more detail, DC27.10 and DC27-CTLA-4 cells and peripheral human T cells were stained with phalloidin-FITC for F-actin and visualized by fluorescence microscopy (Fig. 2). Although resting DC27.10 and DC27.10 CTLA-4 cells were rounded (Fig. 2A, upper right and left panels), anti-CD3/CTLA-4 ligation of DC27.10-CTLA-4 cells for 3 h produced elongated cells with numerous filopodia and lamellipodia (Fig. 2A, lower right panel). This effect was dependent on CTLA-4 since DC27.10 cells failed to elongate (lower left panel). As additional controls, neither anti-CD3 nor anti-CD3/CD28 induced this elongated phenotype (middle panels). The number of cells with filipodia, lamellipodia, or uropods was next scored. Fig. 2B shows examples of each of these structures induced by anti-CD3/CTLA-4 (upper panels a–d). For the filopodia category, only cells with more than five or more filipodia >0.5 μm in length were scored as positive. With DC27.10-CTLA-4 cells, 56% of cells had filopodia in 315 of 560 cells (56%) relative to 9 of 888 cells (1%) for unstimulated cells. An increase in the number of cells with lamellipodia (92 of 560 cells, 16%) or uropod (76 of 560 cells, 13%) was also observed (d). Anti-CD3 or anti-CD3/CD28 had little if any effect on morphology over the time course examined (b and c).

Similar observations were obtained with peripheral CTLA-4-positive T cells with an increase in the presence of filopodia (73 of 143), lamellipodia (30 of 143), and uropods (16 of 143) in anti-CD3/CTLA-4-ligated cells (Fig. 2Cd). Again, anti-CD3 or anti-CD3/CD28 had relatively minor effects (Fig. 2C, b and c). Intracellular staining of CTLA-4 showed no consistent colocalization with lamellipodia, filopodia, and uropods (data not shown). Overall, our findings reveal that anti-CD3/CTLA-4-induced polarization is accompanied by an increase in the presence of filopodia, lamellipodia, and uropods on T cells.

**PI3K, but not PKB/AKT, activity is needed for CTLA-4-induced polarization**

Given the marked effect of CTLA-4 ligation on the morphology of T cells, the next question concerned the nature of the signaling proteins that mediate this event. Candidate proteins such as PI3K, Vav1, and Cdc42 were examined initially. Each of these proteins have previously been implicated in the remodeling of the actin cytoskeleton induced by the AgR (44–46). As we previously described, CTLA-4 has a Tyr-Xaa-Xaa-Met motif that binds to the Src homology 2 domain of the p85 subunit of PI3K (3). The binding avidity is equal to the CD28 pYMNM motif (3). Surprisingly, the loss of the YVKM motif (i.e., DC27.10-CTLA-4 Y201F) failed to interfere with the ability of anti-CTLA-4 to induce cell polarization (Fig. 3A). In both cases, >40% of cells became polarized by 3 h. No difference was noted in the kinetics of the response (data not shown). Despite this, treatment of cells for 1 h with the PI3K inhibitor LY294002 inhibited the change in morphology. The low concentration of LY294002 (10 μM) blocked ~50% of polarization, while 100 μM blocked the event by >95% without having an effect on cell viability. Wild-type and Y201F CTLA-4 cells were affected to a similar extent by the drug. Therefore, while PI3K binding to the coreceptor itself was not needed for polarization, spreading was dependent on general PI3K activity.

Protein kinase B (PKB/AKT) is a major downstream target of the PI3K pathway (47). However, an inhibitor (Src homology 5) of PKB/AKT had no significant effect on anti-CTLA-4-induced cell

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**FIGURE 3.** PI3K but not Akt activity is needed for anti-CD3/CTLA-4-induced cell polarization. A, Polarization requires PI3K activity. PI3K inhibitor LY294002 pretreated DC27.10 CTLA-4 cells or DC27.10 CTLA-4Y201F cells were co-cross-linked with anti-CD3/anti-CTLA-4 for 3 h in the presence of LY294002, and the numbers of polarized cells were scored. Histogram shows the percentage of polarized cells. B, Polarization does not require Akt/PKB activity. Akt inhibitors (SH-5) pretreated cells retained an ability to spread after co-cross-linked with anti-CD3/CTLA-4. Upper panel, Histogram showing the percentage of polarized cells. Lower panel, SH-5 inhibits PKB/AKT activity as shown by an inhibition of anti-phospho-PKB/AKT blotting. Upper panel, Anti-pPKB/AKT blot; lower panel, anti-PKB/AKT protein blot.
Vav-1 is needed for anti-CD3/CTLA-4-induced cell polarization. Vav-1 dominant-negative mutants (L213A GEF mutant and PH domain-deleted mutant) interfere with CTLA-4-induced cell polarization. Vav-1 mutants were transduced into DC27.10 CTLA-4 cells that were then coligated with anti-CD3/CTLA-4 for 3 h. The percentage of polarized cells was then calculated. Myc-tagged Vav-1 mutants expressing levels were measured by Western blot analysis with anti-myc and anti-Vav-1 Ab. A, Microscopic images of cells. a, Medium; b, mock transfected; c, Vav1L213A transfected; and d, Vav1ΔPH domain transfected. Lower panel, Histogram showing the percentage of polarized cells. B, Blot of Vav-1 expression following transfection. Upper panel, Anti-myc blotting for myc-tagged Vav1L213A and Vav1ΔPH domain. Lower panel, Anti-Vav-1 blot against endogenous and transfected Vav1.

Cdc42 is needed for anti-CD3/CTLA-4-induced polarization

Cdc42 is an important member in the Rho family that induces filopodia formation (33). Cdc42 may be activated by Vav-1 GEF activity and plays a central role in the remodeling of the actin cytoskeleton and morphological changes in migration (25, 51). To investigate the link between Cdc42 and CTLA-4 in polarization, DC27.10-CTLA-4 cells were transfected with an inactive form of Cdc42 (Cdc42N17-HA) (52) and constitutively active form of Cdc42 (Cdc42V12-HA) (52), followed by the same procedure to induce cell polarization (Fig. 5). Under these conditions, anti-CD3/CTLA-4-induced polarization was partially impaired (i.e., 50%) by the expression of Cdc42N17 (upper left panel). Roughly, 35–50% of cells were transfected in this assay (data not shown). In addition, anti-CD3 cross-linked Cdc42V12-transfected cells showed an increase in cell polarization (from 12% of mock control to the 25% of Cdc42V12 transfecteds). Anti-CD3/CTLA-4-induced polarization in 55–60% of mock and Cdc42V12-transfected cells. Expression of Cdc42 was confirmed by anti-HA immunoblotting (lower left panel).

To confirm the role of Rho family members, an inhibitor of RhOGTPase, toxin A was also used to pretreat cells for 1 h. This was followed by stimulation with immobilized anti-CD3/CTLA-4, anti-CD3 alone, or anti-CTLA-4 alone. Toxin A inhibits an array of small GTPases, including Rho, Rac, and Cdc42 (53–55). The minimum concentration of toxin A that blocked T cell polarization was 100 ng/ml (lower right panel). This inhibited polarization by 50–60%. The higher concentration of toxin A (1000 ng/ml) completely blocked CTLA-4-induced T cell polarization without any loss in cell viability. These combined data showed inactive Cdc42N17 and toxin A can block anti-CD3/CTLA-4-induced polarization.

Anti-CD3/CTLA-4-induced cell polarization requires MLCK but not ROCK or RhoA

MLCK catalyzes the phosphorylation of myosin L chains, which are involved in actin cytoskeleton rearrangement (56–58). MLCK phosphorylation occurs downstream of Rho GTPases. ML-7 is an inhibitor of MLCK that prevents T cell migration (59). Pretreatment of DC27.10-CTLA-4 cells with ML-7, followed by washing and coligation of anti-CD3 plus anti-CTLA-4 over 4 h, decreased polarization (Fig. 6A). ML-7 inhibited the event by 50% (1 μM), 60% (2 μM), and 70% (4 μM). The data indicated that MLCK activity is needed for CTLA-4-induced cell morphology change. By contrast, neither RhoA nor ROCK appeared to be involved in the event (Fig. 6B). ROCK plays a key role in the regulation of actin-myosin contractility (33, 57), whereas ROCK is activated by RhoA under certain circumstances and regulates detachment of the T cell trailing edge (58, 59). Therefore, we assessed the roles of ROCK and RhoA in anti-CTLA-4-induced cell polarization using the ROCK inhibitor, Y27632 (60), and RhoA inhibitor, C3 enzyme (61). Unlike ML-7 inhibition of MLCK, neither Y27632 (10–100
μM) nor C3 (25–50 μg/ml) interfered with anti-CD3/CTLA-4-induced polarization (Fig. 6B). Most studies in the literature use 10 μM Y27632 and 5 μg/ml C3 to inhibit cell function. Even at a 10-fold higher concentration, no effect of the drugs was observed on anti-CD3/CTLA-4-induced cell elongation. Therefore, although RhoA/ROCK may be found to play a role in the motility of anti-CD3/anti-CTLA-4-activated cells, a role in the polarization of the cells per se was not observed.

Discussion

CTLA-4 has been studied extensively as a negative regulator of T cell activation (4, 5, 62). We previously showed that CTLA-4 can bind to PI3K, up-regulate LFA-1-mediated adhesion, increase motility, and reverse the stop-signal needed for stable T cell interactions with APCs (23, 25). In this study, we show that CTLA-4 ligation, and in particular with TCR/CD3, can induce the rapid polarization of T cells (i.e., within 15–60 min), leading to an increase in numbers of cells with lamellipodia, filopodia, and uropods. This was observed with Ab and CD80 ligand and not with anti-CD3 or anti-CD3/CD28 over the time period examined. We and others (23, 24) previously showed that Rap1 is activated by anti-CTLA-4, while a dominant-negative form of the mediator can block adhesion (23). In this article, we extend the identification of mediators by showing that PI3K, Vav-1, Cdc42, as well as MLCK, are needed for polarization, whereas the mediators PKB/AKT, ROCK, or RhoA are dispensable. Our results therefore indicate that CTLA-4 cosignals have a potent and rapid effect on the polarization of T cells that depends on a specific set of signaling proteins. This event might eventually be found to contribute to CTLA-4 involvement in migration, tolerance induction, and/or autoimmune diseases.

CTLA-4 has been reported to affect numerous aspects of the T cell response (1–5, 62). These include an inhibition of TCR proximal signaling, cytokine production, and entry into the cell cycle. However, the function of the coreceptor is more complicated than previously appreciated. It can bind to PI3K, activate JNK and Rap1, and up-regulate LFA-1-mediated adhesion (8, 9, 17, 23, 24, 63). The increase in JNK activation occurred concurrently with an inhibition of the activation of ERKs (17, 63). The connection to PI3K and Rap1 has been confirmed by several laboratories (3, 7, 23, 24). This range of effects is remarkable given the short cytdomain of CTLA-4 and suggests that alternate molecular mechanisms might account for the function of coreceptor. One additional possible mechanism includes the role of TCR-interacting molecule in shuttling CTLA-4 to the surface of cells (64) and the general effects of increased integrin adhesion and activation of cellular

![FIGURE 5.](http://www.jimmunol.org/)

**FIGURE 5.** Cdc42 is needed for CTLA-4-induced T cell polarization. Cdc42N17-reduced anti-CD3/CTLA-4 coligation induced polarization, whereas Cdc42V12 cooperated with anti-CD3 to induce cell polarization. **Upper left panel,** Histogram showing Cdc42N17 inhibition. **Upper right panel,** Histogram showing anti-CD3 and Cdc42V12 cooperativity. **Lower left panel,** Anti-HA blot showing Cdc42V12 and CdcN17 expression. **Lower right panel,** RhoGTPase inhibitor toxin A inhibited anti-CD3/CTLA-4-induced polarization. Toxin A was used to pretreat (0.5 h) DC27.10 CTLA-4 cells, followed by coligation with anti-CD3/anti-CTLA-4 for 3 h. Histogram showing percentage of polarized cells.

![FIGURE 6.](http://www.jimmunol.org/)

**FIGURE 6.** CTLA-4-induced cell polarization requires MLCK but not ROCK and RhoA. A, MLCK inhibitor (ML-7)-pretreated DC27.10 CTLA-4 cells were coligated with anti-CD3/anti-CTLA-4 for 3 h, and the percentage of polarized cells was calculated. Histogram showing the percentage of polarized cells. B, RhoA inhibitor (C3 enzyme) and Rock inhibitor (Y27632) treated cells retained the ability to polarize after coligation with anti-CD3/CTLA-4. Histogram showing the percentage of polarized cells.
pathways (23). Clearly, an understanding of CTLA-4 function will depend on uncovering the full range of positive and negative signals to regulate its function.

This study extends this theme of increased integrin adhesion and motility by showing that CTLA-4 has an unexpectedly rapid and potent in inducing polarization with increased numbers of lamel-lipodia, filopodia, and uropods (Figs. 1 and 2). The increased polarization is entirely consistent with our previous observations of increased motility, whereas the rapidity of the altered morphology was unexpected. Spreading was detected as early as 15 min post-ligation, with 50% of cells showing an elongated phenotype by 2–3 h. Both anti-CTLA-4 and CD80-Ig induced the effect over a titration. This was observed in T cell hybridoma and preactivated CTLA-4-positive human T cells. Anti-CTLA-4 alone induced some polarization, although less effective than in conjunction with TCR-CD3 signals. CTLA-4 can cooperate with anti-CD3 to amplify polarization. The effect was seen with soluble and immobilized ligands but was more rapidly induced in the DC27.10 hybridoma (i.e., minutes) than peripheral T cells (i.e., hours). Neither anti-CD3 alone or anti-CD3/CD28 was able to manifest these effects over the time course examined. Anti-CD3 normally induces this change only after some 12–24 h of culture (65). Although most of the experiments in this article did not involve the use of exogenous ligands such as ICAM-1, FCS in the culture medium contains fibronectin that can bind to plates and serve as ligand for integrins.

To our knowledge, the potency and rapidity of the polarization effect has not been observed previously with the ligation of T cell surface receptors. The one exception that concerns chemokines where stromal cell-derived factor-1α and CXCL8 can induce polarization by as early as 5–30 min (66, 67). Whether CTLA-4 and chemokine receptors share a target in generation of signals remains to be determined. The ability of CTLA-4 to induce polarization is consistent with its effect on motility (25) and may eventually be found to influence events such as cell migration and T cell homing (27, 28).

Given CTLA-4 induction of T cell polarity, the identity of mediators needed to be identified, and whether CTLA-4 uses the same or different mediators than described in other systems. As mentioned, Rap1 is one mediator that is activated by anti-CTLA-4 (23, 24). Constitutively active Rap1 can cooperate with anti-CD3 ligation to increase ICAM-1 binding while a dominant negative of the mediator can block CTLA-4 induced adhesion (23). Because it is needed for the up-regulation of LFA-1 adhesion, it is consider a precursor step for polarization. In other systems, polarization can be mediated by PI3K (34, 36, 68) and Rho family GTPases (69). Consistent with this, anti-CD3/CTLA-4-induced polarization was inhibited by an inhibitor of PI 3K (Fig. 3), although two unexpected findings followed. First, the YXXM motif (i.e., CTLA-4-Y201F) mutant readily induced polarization at levels comparable to the wild-type receptor (Fig. 3A). The dependency on PI3K occurred independently of direct PI3K binding to the cytoskeleton. In this case, anti-CD3 may suffice to generate adequate amounts of D-3 lipids. Associated PI3K may nevertheless serve as a back-up to ensure the adequate production of D3 lipids, especially with low-affinity peptide where the TCR would be expected to induce low levels of phosphatidylinositol 4,5-bisphosphate or PIP2. Second, it was interesting that the inhibition of AKt/PKB did not affect the induction of cell polarity (Fig. 3B). This kinase is a prime downstream target of PI3K and yet played no obvious role in the induction of morphology changes. This underscores the specificity of different pathways linked to a single receptor in the regulation of different cellular events. PKB phosphorylates other substrates such as glycogen synthase kinase-3 (GSK-3) and regulates other functions such as protein synthesis, metabolism, and cell cycle (70).

Another possible target of the PI3K pathway was Vav-1 (47, 71). Although the exact upstream vs downstream role of PI3K and Vav-1 is unclear, Vav-1 GEF activity has been reported to depend on inositol lipids (44). Others have found a role for Vav-1 in activating PI3K (45). Phosphatidylinositol 4,5-bisphosphate or PIP2 binding to PH domain of Vav-1 may modulate GEF activity (44). By contrast, the inhibition of PI 3K does not inhibit Vav-3 phosphorylation in Vav-3-deficient cells (72), while BeC and TCR activation of PKB occurs normally in Vav-1-deficient B cells (73). Recently, we showed that TCR and TCR/CD28 engagement of PKB/Akt and GSK-3 in T cells can operate independently of Vav-1 (74). Vav-2.3 could partially substitute for the absence of Vav-1, but the PKB-GSK-3 pathway was still partially operative in the absence of all three Vav isoforms (74). We showed that dominant-negative forms of Vav-1 with mutations in either the Dbl homology and PH domains (Vav-1GEF* and Vav-1ΔPH) inhibited CTLA-4-induced polarization (Fig. 4). Mice bearing the Vav-1 mutation of PH domain (Vav-1E(G225R)) decreased TCR-induced Vav-1 GEF activity (45). The inhibition of adhesion was partial, either reflecting the partial involvement of the protein, or the fact that the transfection efficiency was 35–50% (data not shown), or the possibility that Vav-2 and 3 may substitute for Vav-1 in the regulation of this event. Consistent with this role, Vav-1 localizes at leading edge and the trailing edge or uropod (65).

Further along this pathway, Vav-1 exchange activity has been implicated in the activation of Rho family members such as Rac1 and Cdc42 (46, 47). In turn, Cdc42 is the main regulator of filopodia formation (33, 75). Cdc42 affects cell polarization by localizing the microtubule-organizing center and Golgi apparatus in the front of the nucleus (25). It has been reported that the activation of Cdc42 in Vav-1−/− T cells is defective (46). Cdc42 activity has been implicated in phosphorylation and tension homolog deleted on chromosome 10 exclusion from protrusion in leukocytes and PIP3, appears to be required for localizing Cdc42 activity (37, 76). Consistent with this, our data showed that both Clostridium difficile toxin A and the inactive form of Cdc42 (Cdc42N17) abrogated the CTLA-4-induced T cell polarization (Fig. 5). In addition, active form of Cdc42 (Cdc42V12) appeared to cooperate with TCR to increase the percentage of the polarization cells when compared with TCR signal alone. Overall, our data indicate that PI3K, Vav-1, and Cdc42 are needed for anti-CD3/CTLA-4-induced T cell polarization.

The last connection between CTLA-4 and morphology concerned MLCK. MLCK was been proposed to regulate actin-myosin-mediated shape change leading to cell-cell contact. Further work is needed to determine what stage MLCK regulates CTLA-4 signaling.

The biological role of CTLA-4 in regulating pathogenesis is extensive. CTLA-4-deficient mice develop a fatal lymphoproliferative disorder and die at 18–28 days of age due to lymphocytic infiltration into nonlymphoid tissues (5, 62). There is evidence for the role of CTLA-4 in autoimmune endocrinopathies, including Graves’ disease, autoimmune hypothyroidism, and type 1 diabetes mellitus (77). Also, blockade of CTLA-4 can increase experimental autoimmune encephalomyelitis (6). Future studies will be needed to assess the role of polarization and motility on lymphocytic migration (i.e., affecting tissue infiltration) during anergy induction and in the regulation of tolerance.
Disclosures

The authors have no financial conflict of interest.

References


