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CTLA-4 disrupts ZAP70 microcluster formation with reduced T cell/APC dwell times and calcium mobilization

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Abstract

CTLA-4 is a co-receptor that modulates the threshold of T cell activation and autoimmunity. We previously showed that CTLA-4 reverses the TCR-mediated stop signal needed for T cell/APC interactions [Schneider *et al.*, *Science* 2006, 313: 1972]. In this study, using a different T cell system, we show that CTLA-4 expression changed the behavior of T8.1 T cells by reducing the contact time between T cell and APC, preventing re-inforced contacts, and reducing the contact area at the immunological synapse. This led to a major reduction in Ca²⁺ influx/mobilization and interleukin-2 production. Further, anti-CD3/CTLA-4 increased T cell motility on antibody-coated glass slides, concurrent with an abrogation of ZAP70 microcluster formation. Our findings further support a role for CTLA-4 in limiting the interaction between T cell and APC that is needed for optimal activation.

Keywords

Conjugation; CTLA-4; Intracellular calcium; ZAP70 microclusters

Introduction

CD28 and CTLA-4 binding to CD80/86 on antigen-presenting cells (APC) play central roles in determining the outcome of T cell activation [1–4]. CTLA-4 binds with higher avidities than CD28 and bivalent CTLA-4 dimers can bridge bivalent CD80 homodimers [5]. CTLA-4 deficient (*Ctla-4*^{-/-}) mice show a massive lympho-proliferative disorder, increased numbers of activated T cells and autoimmune disease with organ destruction [6, 7]. Similarly, lentiviral-induced siRNA mice have shown a concurrent reduction in CTLA-4 and a more rapid onset of diabetes [8]. Co-receptor expression is needed for anergy induction [9]. In this manner, CTLA-4 can modulate the threshold for T cell activation where it has been linked to the onset of autoimmune disorders such as type-1 diabetes [10].

Several mechanisms have been proposed to account for the molecular mechanism by which CTLA-4 generates inhibitory signals. These include ectodomain competition for CD28

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binding to CD80 and CD86 [11], disruption of CD28 localization at the immunological synapse (IS) [12], modulation of phosphatases PP2A and SHP-2 [13–15] and interference with lipid raft expression [16–19]. CTLA-4 engagement of CD80 and CD86 on dendritic cells can induce the release of indoleamine 2,3-dioxygenase [20, 21].

Recently, we demonstrated that anti-CTLA-4 can reverse the anti-TCR stop signal for motility, and that CTLA-4-positive primary cells show reduced contact times with APC [22]. In this study, we extend this model by showing that the mere expression of CTLA-4 can markedly change the interaction between T cells and APC. Using a T cell hybridoma reactive to tetanus toxin (Ttox) peptide, we show that the expression of CTLA-4 in T cells reduced the T cell/APC dwell time, prevented the formation of an enforced contact region with APC (*i.e.* instead cells remained tethered) and reduced the contact area at the IS. This in turn led to a major reduction in Ca^{2+} influx/mobilization and IL-2 production. Further, we show for the first time that anti-CD3/CTLA-4 abrogated anti-CD3-induced ZAP70 microcluster formation, while increasing motility on antibody-coated glass slides. Our findings further support a role for CTLA-4 in limiting T cell/APC conjugation as well as preventing microcluster formation needed for T cell activation.

Results and discussion

CTLA-4 interferes with long-term T cell/APC conjugation

T8.1 cells express human CD4 and a chimeric human-mouse TCR specific for Ttox (830–843) peptide, and lack expression of CTLA-4 [23]. The TCR reacts to Ttox peptide as presented by L625.7 cells expressing the appropriate MHC (HLA-DR*1102) and CD80/86. These cells have been used to demonstrate a requirement for Lck in T cell/APC conjugation and T cell polarization [24]. The transfectants express low levels of surface CTLA-4 without an effect on CD28 and TCR expression, as previously described [25, 26]. Using *in vitro* migration assays and *in vivo* two-photon microscopy, we previously reported that anti-CTLA-4 over-rides the TCR/CD3-induced stop signal for motility [22].

To assess the interaction between T cells and APC in more detail using a different cell system, T8.1 WT and T8.1-CTLA-4 cells were incubated with Ttox peptide-loaded L625.7 cells and monitored for *in vitro* conjugation (Fig. 1A). T cells normally undergo various changes in their interaction with APC – an initial tethered-like contact over the first 1–2 min followed by an enforced contact with spreading at the contact region between the T cell and APC at 2–10 min [27, 28]. Individual T cells were monitored for the duration and frequency of an interaction over a 1200-s incubation period.

The first observation was that while 60% of WT cells underwent initial contact followed by an enforced contact with APC, only 10–15% of T8.1-CTLA-4 cells underwent enforced contact with APC over the 20-min incubation period (upper panel). An example of T cells developing an enforced contact at 0–80 s with APC is seen in the upper right panels. Cells with a T cell/APC contact greater than 12 μm were designated as having enforced contact. Conversely, some 15% of WT cells remained tethered without evidence of enforced contact (lower panel). By contrast, 40–45% of T8.1-CTLA-4 cells became tethered (lower panel). Cells with a T cell/APC contact less than 12 μm were designated as tethered cells. Examples

of T cells with a tethered APC contact are seen in the images at 0 and 80 s in the lower right panels. These cells would often continue to move circuitously at the point of contact with the APC.

A direct measurement of the maximum length of the T cell/APC contact area confirmed that WT cells formed a more extended region of contact with APC than cells expressing CTLA-4 (*i.e.* 18 μm vs. 10 μm) (Fig. 1B). Similarly, WT cells showed longer-term interactions with APC than T8.1-CTLA-4 cells (Fig. 1C). Dot plot analysis showed heterogeneity in the length of time individual cells interacted with APC (*i.e.* from 100 to 1000 s); however, the mean time of interaction of T8.1 WT cells with APC was 540 s compared to 180 s for T8.1-CTLA-4 cells. These observations indicate that the mere expression of CTLA-4 alters the ability of T cells to stably interact with APC, a result consistent with our previous report documenting a reversal of the TCR stop signal by CTLA-4 in the same cells [22].

CTLA-4 effects on reduced T cell/APC dwell times result in reduced Ca^{2+} influx/mobilization and IL-2 production

The next question was whether reduced stability of T cell adhesion to APC resulted in a reduced response to Ttox (Fig. 2). For this, Fura-1/AM-loaded T8.1 WT and T8.1-CTLA-4 cells were incubated with Ttox-pulsed L625.7 cells and monitored for an increase in intracellular calcium. Transmitted light images were measured in 10-s intervals for intracellular calcium (340 and 380 nm excitation, 510 nm emission) (Fig. 2; Supporting Information Fig. 1, movies). While the addition of Ttox induced a significant Ca^{2+} response over 1500 s (Fig. 2A, upper movie panel and lower panel), little response was detected in T8.1-CTLA-4 cells (Fig. 2B, upper and lower panels). The level was only slightly above the basal calcium level. These data clearly indicated that the mere expression of CTLA-4 resulted in a major reduction in the rise of intracellular calcium that correlated with the less stable or shorter dwell times between T8.1-CTLA-4 cells and APC. Further correlated with this observation was a reduction in IL-2 production in response to Ttox at 24 h (Fig. 2C), which was also observed at 48 h (data not shown).

CTLA-4 co-ligation markedly reduces ZAP70 microcluster formation induced by anti-CD3

To assess further the function of CTLA-4 in a different context, we monitored the behavior of T8.1 WT and T8.1-CTLA-4 cells on antibody-coated glass slides as described by others [29]. For this, antibodies were attached to the surface of slides followed by the overlaying of settling cells and their observation at the interface between cell and slide surface [29]. In the presence of anti-CD3, T cells (T8.1 WT and T8.1-CTLA-4) underwent little movement when compared to resting cells (Fig. 3A, lower left histogram). By contrast, anti-CD3/CTLA-4 co-ligation allowed for extensive movement of T8.1-CTLA-4 cells. While anti-CD3-ligated cells moved slowly at less than 4 $\mu\text{m}/\text{min}$, anti-CD3/CTLA-4-ligated T8.1-CTLA-4 cells achieved velocities of 20 $\mu\text{m}/\text{min}$. Examples of the movement of individual cells are shown in XY tracker charts (upper and right panels). Serum proteins such as fibronectin may also contribute to motility. This observation confirms, using a different system (*i.e.* T8.1 cells on antibody-coated slides), that CD3/CTLA-4 can reverse the stop signal for T cell motility.

We next then examined the well-documented ability of anti-CD3 to induce ZAP70 microcluster formation in T cells (Fig. 3B). Recent studies have documented the importance of anti-CD3-induced microcluster formation for T cell signaling [29, 30]. T8.1 WT and T8.1-CTLA-4 cells were transfected with mRFP-ZAP70 and monitored at the interface for microcluster formation using a Zeiss LSM 510 confocal microscope. Cells were monitored continuously for 7–350 s following the initial contact with the slides.

mRFP-ZAP70 was diffusely distributed throughout unligated T8.1 WT and T8.1-CTLA-4 cells (top panels). Within seconds of exposure to anti-CD3, ZAP70 microclusters formed in the majority of T8.1 WT and T8.1-CTLA-4 cells (*i.e.* 80–90%) (upper middle panels; right histogram). At the earlier time points (*i.e.* 1–100 s), the ZAP70 microclusters were located mostly at the periphery of the cells with limited movement to the center, after which they began to disappear, as previously reported [29–31]. By contrast, anti-CTLA-4 co-ligation with anti-CD3 markedly reduced ZAP70 microcluster formation in T8.1-CTLA-4 cells (lower middle right panel; histogram).

Of the 5–10% of cells that still showed some microcluster formation, both the number and size of microclusters were reduced. The majority of cells showed a pattern of diffusely dispersed mRFP-ZAP70 throughout the cell (*i.e.* less intense signal due to dispersion). The same pattern was observed in resting cells (top panels) and in cells ligated alone with anti-CTLA-4 (lower panels). The movement of individual microclusters within the peripheral region as shown in the XY tracker charts showed similar degrees of movement in T8.1 WT and T8.1-CTLA-4 cells (lower graphs). These findings indicated using a different system (*i.e.* antibodies on slides) that CTLA-4 co-ligation over-rides the anti-CD3 stop signal and further there is a marked affect in reducing ZAP70 microcluster formation.

Overall, although we previously showed that anti-CTLA-4 reverses the TCR/CD3 mediated stop signal [22], an important issue had been to determine whether this occurs in other model systems, and whether the simple expression of CTLA-4 in a given T cell can reproduce these effects. The use of blocking reagents has been complicated by the ability of CD80/86 to bind to CD28/CTLA-4/programmed death 1 ligand [32] and the uncertain effects of mono- and bivalent antibodies on CTLA-4 function. The simplicity with T8.1 WT and T8.1-CTLA-4 cells is that they differ only by the expression of CTLA-4.

From this, we showed that CTLA-4 expression causes a major change in the behavior of T8.1 T cells in the context of multiple parameters. CTLA-4 reduced the dwell times with APC and prevented the formation of reinforced contacts at the IS (Fig. 1). Instead, these cells made initial contact with APC, but continued to move and failed to form a stable interface as was observed with WT cells (Fig. 1A). They generally remained tethered, often dangling at the contact point with APC, as described with tolerized T cells [33, 34]. This in turn was correlated with a major reduction in calcium mobilization and IL-2 production (Fig. 1, 2). While an effect in early signaling events could contribute to the impairment of conjugation, the reduction in dwell times and re-inforced contact between T cells and APC also correlated well with the major impairment of calcium mobilization and cytokine production.

In addition to these effects, we showed for the first time that anti-CTLA-4 co-ligation had a major effect on the ability of anti-CD3 to induce microcluster formation (Fig. 3). Microclusters form shortly after the TCR ligation and involve the TCR complex as well as signaling mediators such as ZAP70, Src homology 2 domain-containing leukocyte protein of 76 kDa (SLP-76) and GADS. Some of these mediators exist in overlapping clusters (*i.e.* GADS and SLP-76), while other complexes carrying ZAP70 can transiently interact with the TCR or SLP-76 clusters [29, 30]. Peripheral microclusters can sustain signaling that is terminated in the central supramolecular activation cluster [35].

Our findings clearly showed that CTLA-4 co-ligation blocked ZAP70 microcluster formation in the majority of cells from the earliest time point and throughout the incubation period (*i.e.* 5–10 min). The pattern of mRFP-ZAP70 in the CTLA-4-ligated cells was comparable to the pattern observed in resting cells where the signal was evenly dispersed throughout the cells. The co-receptor therefore has an unusually potent ability to disrupt the earliest of TCR-mediated events needed for the successful propagation of signals required for the stimulation of cells. Whether this is due to the engagement of intracellular negative regulator, and/or is the result of continuous motility, is not clear. The continuous motility induced by CTLA-4 may not permit stable enough interactions with antibody on plates to allow for microcluster formation. This may be related to the steric effects of motility on stable TCR aggregation (*i.e.* the need for the stop signal) and re-directed signaling. Nevertheless, the ability to over-ride the stop signal is consistent with the observed effect of the coreceptor in limiting conjugation times, inhibiting ZAP70 microcluster formation as well as calcium mobilization in the antigen presentation system. These effects are consistent with a role for CTLA-4 in energy and tolerance induction [9]. Although the basis for the effect of anti-CTLA-4 on motility is not known, the polarization involves phosphatidylinositol 3-kinase, Vav-1, Cdc42, and myosin light chain kinase [36].

While there are advantages and disadvantages in examining CTLA-4 function in T8.1 cells relative to other cells, one advantage was the lack of involvement of regulatory T cells (Treg). None of the T8.1 cells express Foxp3 (data not shown) and yet they behave in ways comparable to separated CTLA-4-positive primary T cells that include the possible presence of Treg [37, 38]. Our findings indicate that CTLA-4 can influence T cell/APC interactions and motility independently of Treg involvement. Future studies will be needed to establish whether the formation of other microclusters is affected by CTLA-4, and relative effects of intracellular signaling *vs.* motility on the process.

Concluding remarks

Our previous studies have shown that T cell motility and the TCR-induced stop signal can be reversed by CTLA-4 [22]. Our present study shows that the mere expression of CTLA-4 on T8.1 cells in the context of CD80/CD86-expressing APC markedly changed the ability of T cells to form stable, enforced contacts with APC and reduced the contact area at the IS as well as Ca²⁺ influx/mobilization and IL-2 production. Further, anti-CD3/CTLA-4 co-ligation by antibody on glass slides led to increased cell motility and disrupted ZAP70 microcluster formation that was otherwise induced by anti-CD3. Collectively, these findings

further support the notion that CTLA-4 limits the dwell times and the formation of microclusters needed for optimal T cell activation.

Materials and methods

Cells and reagents

The murine hybridoma T8.1 expresses a chimeric human-mouse TCR specific for Ttox (830–843) peptide restricted by HLA-DRB1*1102. The DR-expressing murine fibroblasts L625.7 used as APC also express endogenous B7-1/2 and ICAM-2 (gifts from O. Acuto, Oxford University, Oxford, UK). Stable T8.1-CTLA-4 transfectants were generated using the human CTLA-4 gene in the pSRa vector together with pBABE containing a puromycin-resistant gene [25]. Cells were maintained in DMEM with 10% FCS, 2 mM L-glutamine, supplemented with 400 nM methotrexate, 1 mg/mL G418, and 50 μ M 2-ME. L625.7 cells were cultured in complete DMEM containing 250 μ g/mL G418. Anti-mouse CD3 (145-2C11) was obtained from American Type Culture Collection. Anti-human CTLA-4 (BNI3) was kindly provided by B. Broeker (Greifswald, Germany). Poly-L-lysine was purchased from Sigma, chambered coverglass from Nalge Nunc. pMXs-mZAP70-mRFP was a kind gift from T. Saito (Japan).

Single-cell calcium measurements and video imaging

L625.7 fibroblasts (3.5×10^5 /mL) pulsed with 1 μ g/mL of Ttox (830–843) peptide were plated in their culture medium on glass coverslips mounted in 25-mm Petri dishes and incubated at 37°C. T8.1 WT and T8.1-CTLA-4 cells (3.5×10^5) were incubated for 20 min at 37°C with 1 μ M Fura-2/AM in 1 mL of 10 mM HEPES buffer, pH 7.5, containing 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 1 mM Na₂HPO₄ and 1 mg/mL glucose, washed and added to fibroblasts in a final volume of 200 μ L. After 2 min to allow the T cells to deposit, acquisition was performed at 37°C on an Eclipse TE300 inverted microscope as mentioned above. Transmitted light images were taken every 10 s in turn with intracellular calcium measurement (340 and 380 nm excitation, 510 nm emission) images. Movies were made with the Metafluor software.

T cell/APC conjugation

L625.7 and T8.1 cells were treated and cultured as described above. Transmitted light images were acquired every 10 s during 20 min. T cell/APC interactions were then monitored by ImageJ software.

Imaging of cells on antibody-coated chambered glass culture slides

Poly-L-lysine-treated chambered glass culture slides were coated with 5 μ g/mL anti-CD3 and/or 20 μ g/mL anti-CTLA-4 antibodies for 1 h at 37°C to generate antigenic surfaces. Nonspecific binding sites on the glass surface were blocked with RPMI 1640 containing 10% FCS for 10 min prior to imaging. Cells were imaged for the interface using a Zeiss LSM 510 confocal microscope. The temperature of the sample was maintained at 37°C.

IL-2 measurement

T8.1 WT and T8.1-CTLA-4 cells were co-cultured with L625.7 cells (1:1) pulsed with different concentrations of Ttox peptide. After 24 h, IL-2 production was measured by ELISA (PharMingen).

Statistical analysis

Results are given as the mean \pm the standard error of the mean. Statistical analysis was performed using either two-tailed unpaired Student's *t*-test or one-way ANOVA using GraphPad Prism; *p* value <0.05 was considered as significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IS	immunological synapse
SLP-76	Src homology 2 domain-containing leukocyte protein of 76 kDa
Ttox	tetanus toxin

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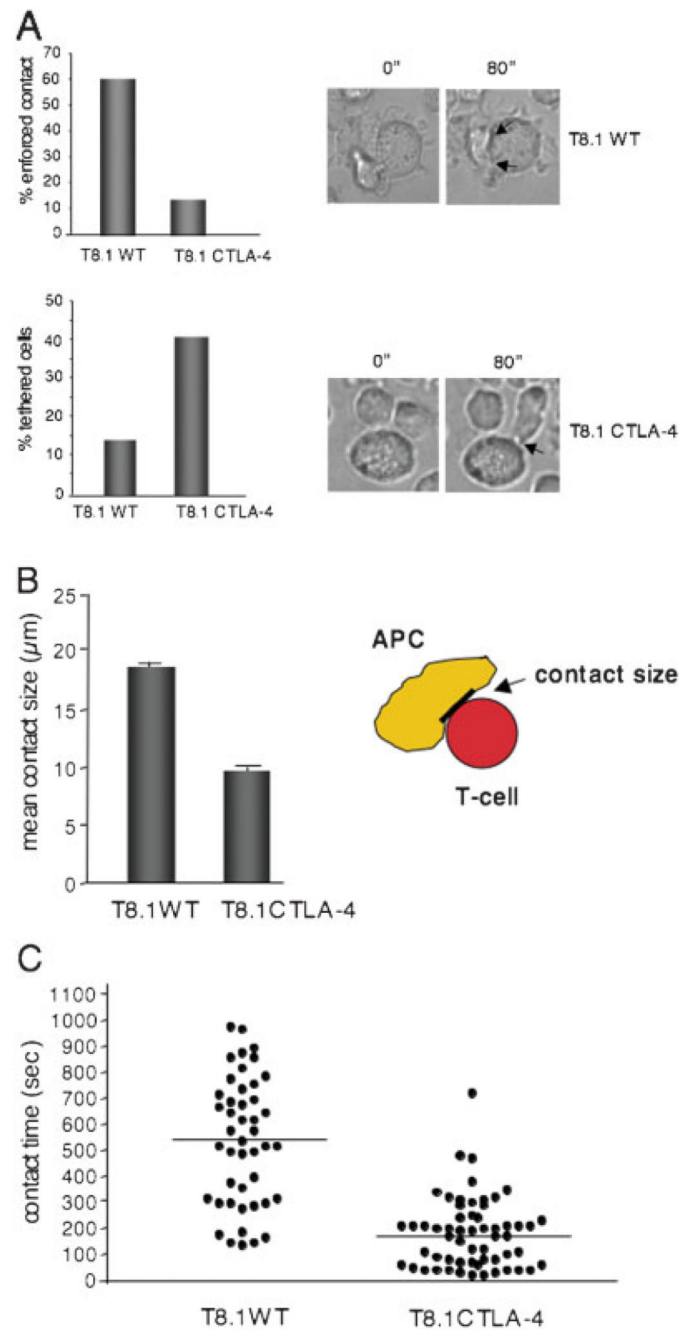
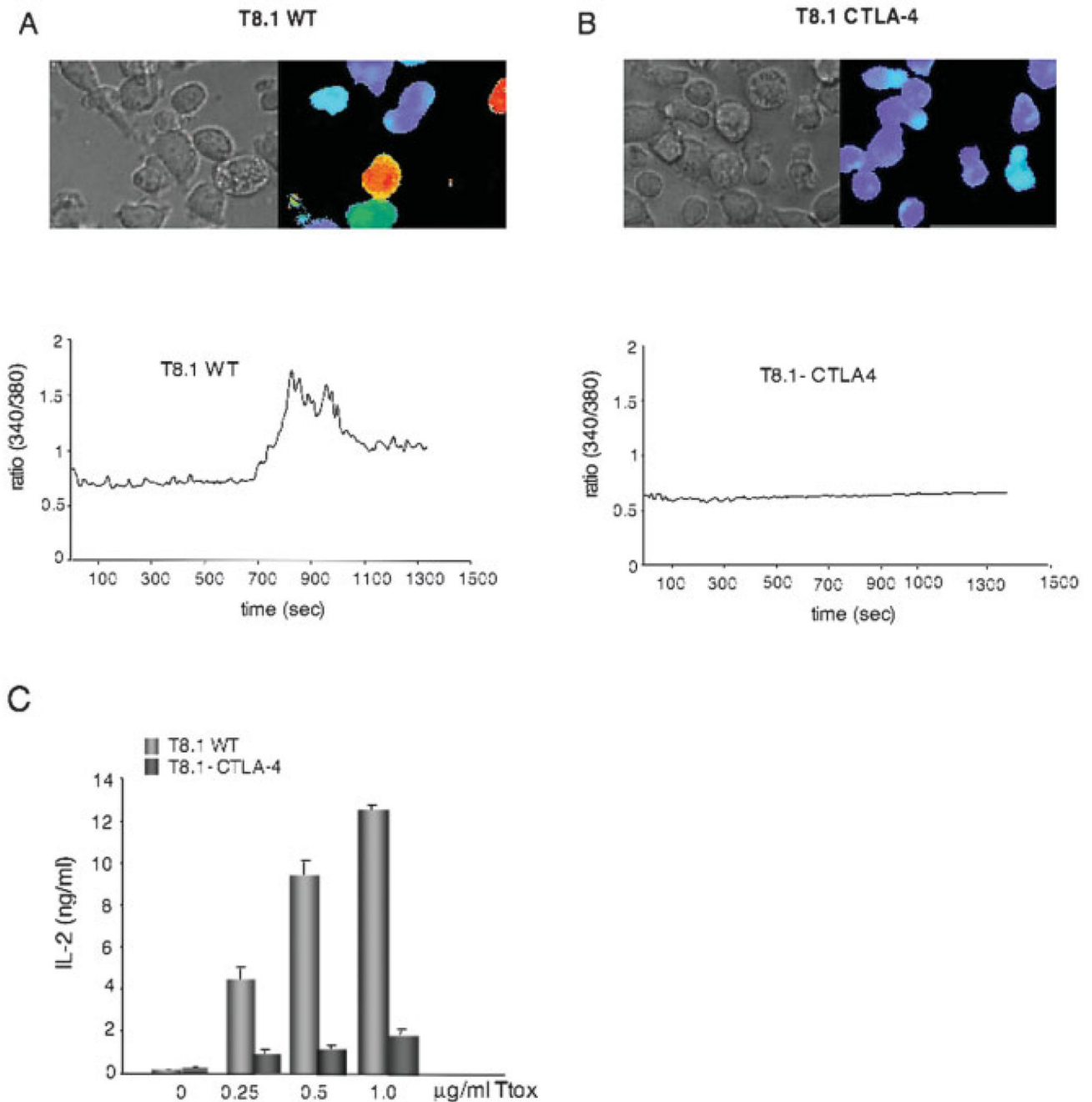


Figure 1.

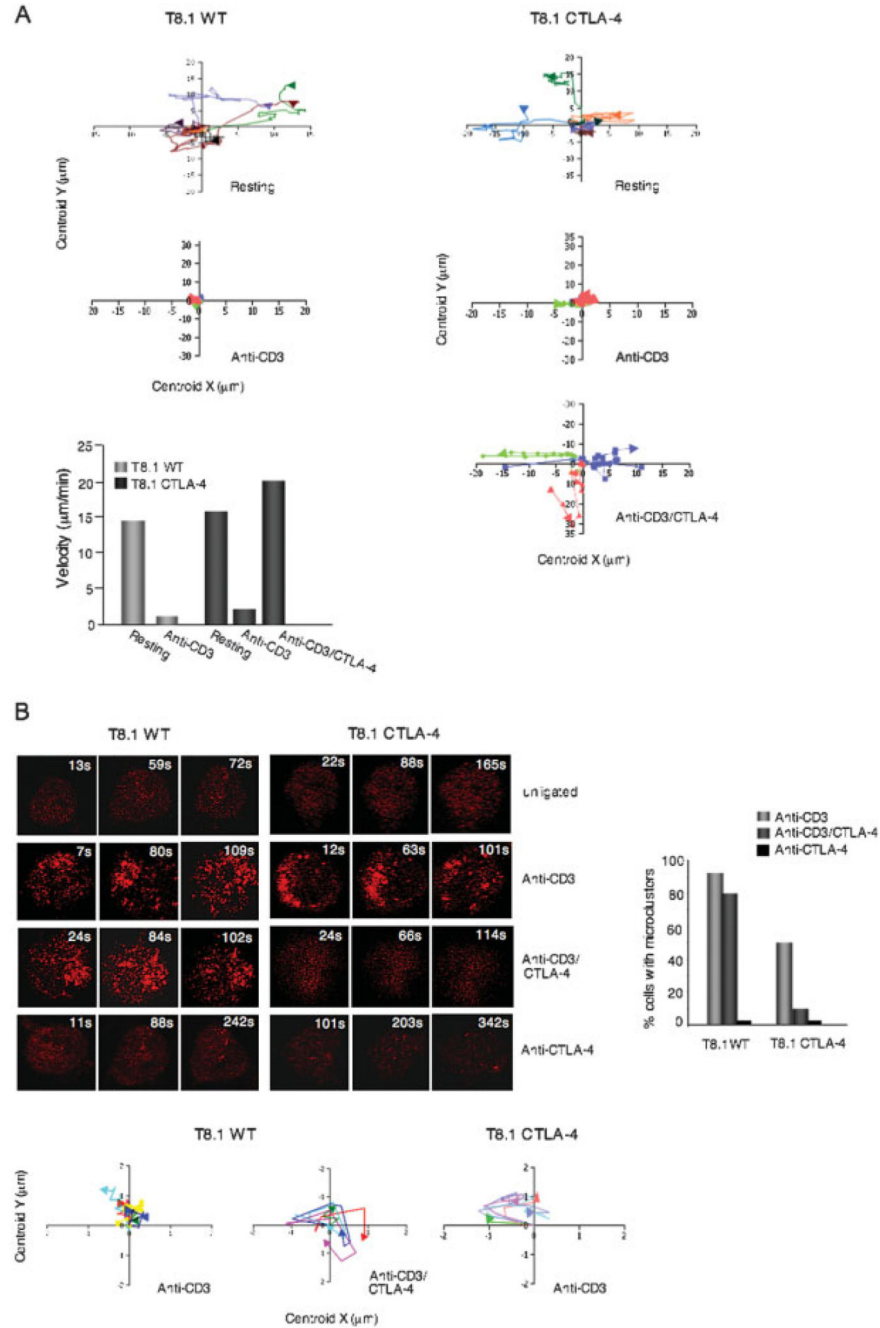
CTLA-4 expression alters T8.1 T cell adhesion to APC. (A) T8.1-CTLA-4 cells fail to undergo enforced contact with APC. T8.1 WT and T8.1-CTLA-4 cells were incubated with L625.7 cells pulsed with Ttox (830–843) peptide and more than 100 cells were analyzed for tethered vs. stable interactions with APC. Upper panel, left: histogram showing the percentage of cells with enforced contact with APC. Upper panel, right: light transmission images of a typical conjugate formed between T8.1 WT cells and an APC, showing enforced contact. Lower panel, left: histogram showing the percentage of cells with tethered contact

with APC. Lower panel, right: light transmission images of a typical conjugate formed between T8.1-CTLA-4 cells and an APC, showing a tethered contact. (B) T8.1-CTLA-4 and T8.1 WT cells differ in the size of the contact area at the IS. T8.1 WT and T8.1-CTLA-4 cells were incubated with L625.7 cells pulsed with Ttox (830–843) peptide and more than 100 cells were analyzed for the size of the contact area with APC. (C) T8.1-CTLA-4 cells show reduced contact times with APC. T8.1 WT and T8.1-CTLA-4 cells were incubated with L625.7 cells pulsed with Ttox (830–843) peptide (1.0 $\mu\text{g}/\text{mL}$) and at least 50 cells were monitored for the duration and frequency of binding to APC over a 1200-s incubation period. Similar results were obtained from two other independent experiments.

**Figure 2.**

CTLA-4 expression reduces calcium mobilization concurrent with impaired interaction with APC. (A) T8.1 WT cells increase intracellular Ca^{2+} in response to antigen. L625.7 cells pulsed with 1 $\mu\text{g}/\text{mL}$ of Ttox (830–843) peptide were incubated with 1 μM Fura-2/AM-loaded T8.1 WT cells. Transmitted light images were taken every 10 s in turn with intracellular calcium measurement (340 and 380 nm excitation, 510 nm emission; pseudocolor images corresponding to 340/380 ratios). Shown are typical images extracted from movies (see Supporting Information Fig. 1). Mean calcium responses of several

individual cells are shown in the lower panels for T8.1 WT ($n=39$) and T8.1-CTLA-4 cells ($n=64$). Only cells making contacts with conjugates were analyzed. Upper panel: movies showing the change in Fura-2/AM color emission for T8.1 WT cells. Lower panel: figure showing the analysis of change in levels of intracellular Ca^{2+} . (B) T8.1-CTLA-4 cells show impaired Ca^{2+} response to antigen. L625.7 cells pulsed and incubated with T8.1-CTLA-4-positive cells as described above. Upper panel: movies (see Supporting Information Fig. 1) showing the change in Fura-2/AM color emission for T8.1 WT cells. Lower panel: figure showing the analysis of change in levels of intracellular Ca^{2+} . (C) T8.1-CTLA-4 cells show impaired IL-2 production in response to Ttox. T8.1 WT and T8.1-CTLA-4 cells were co-cultured with L625.7 cells (1:1) pulsed with different concentrations of Ttox peptide. After 24 h, supernatants were taken and IL-2 production was measured by ELISA.

**Figure 3.**

CTLA-4 co-ligation increased cell motility on antibody-coated glass slides while reducing the formation of ZAP70 microclusters. (A) Upper left and right panels: XY tracker charts showing examples of movement of individual microclusters formed in T8.1 WT and T8.1-CTLA-4 cells when left untreated or stimulated with anti-CD3 or anti-CD3/CTLA-4. Lower left panel: T8.1-CTLA-4 cells show increased motility. T8.1 WT and T8.1-CTLA-4 cells transfected with mRFP-ZAP70 were plated in their culture medium on glass slides coated with anti-CD3 or anti-CD3/CTLA-4 and monitored for movement using a Zeiss LSM 510

confocal microscope. Velocity software was used for measuring velocity. (B) Left upper panel: T8.1-CTLA-4 cells show defective ZAP70 cluster formation in response to anti-CD3/CTLA-4 ligation. T8.1 WT and T8.1-CTLA-4 cells transfected with mRFP-ZAP70 were plated in their culture medium on glass culture slides coated with anti-CD3, anti-CD3/CTLA-4 or anti-CTLA-4. Cells were imaged for the formation of ZAP70 microclusters at the interface for a period of time using a Zeiss LSM 510 confocal microscope. Right panel: histogram showing the percentage of cells forming ZAP70 microclusters. Left lower panels: XY tracker charts showing microcluster movement of T8.1 WT cells treated with anti-CD3 or anti-CD3/CTLA-4, and T8.1-CTLA-4 cells treated with anti-CD3. Similar results were obtained from at least two other independent experiments.