Altered proximal T cell receptor (TCR) signaling in human CD4+CD25+ regulatory T cells

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Abstract: CD4+CD25+ regulatory T cells play an important role in peripheral tolerance. Upon T cell receptor (TCR)-mediated activation, the cells fail to proliferate but are induced to have a suppressor function. The intracellular signaling events that lead to their responses have not been elucidated. In this study, we have examined the proximal TCR signaling events in freshly isolated human CD4+CD25+ regulatory T cells after TCR ligation. In contrast to CD4+CD25− T cells, TCR ligation of CD4+CD25+ regulatory T cells by anti-CD3 cross-linking resulted in a lower calcium influx and extracellular signal-regulated kinase 1/2 phosphorylation. Examination of the CD3ζ chain phosphorylation status indicated that CD4+CD25+ regulatory T cells have poor phosphorylation of the protein and consequently, reduced recruitment of ζ-associated protein-70 to the TCR immunoreceptor tyrosine motif. The adaptor protein, Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa, which relays signals to downstream signaling components, also showed reduced phosphorylation, which correlated with reduced VAV guanine nucleotide exchange factors association. Consistent with other findings, the defect is accompanied with impaired actin cap formation, implicating a failure of actin remodeling of the cells. Together, our results demonstrate that CD4+CD25+ regulatory T cells have altered TCR proximal signaling pathways, which could be critical for inducing the distinct behavior of these cells. J. Leukoc. Biol. 80: 145–151; 2006.

Key Words: T cell activation · signal transduction · immunoregulation

INTRODUCTION

CD4+CD25+ regulatory T cells comprise 5–15% of peripheral CD4+ T cells. They are capable of suppressing the proliferation of responder T cells in vitro and inhibiting a variety of autoimmune, inflammatory, and alloreactive responses [1–3]. The precise mechanisms of immunoregulation by CD4+CD25+ T cells remain to be defined. In vitro analyses have suggested that the suppression is a cell-to-cell, contact-dependent, and cytokine-independent process [1–3]. The intracellular signals that are required for the suppression have not been elucidated fully. T cell activation does not induce CD4+CD25+ T cell proliferation but instead, is necessary to induce their suppressive properties [4, 5]. Once they are activated, they can suppress T cell responses in an antigen-nonspecific manner. Full T cell activation requires the presence of two signals. The first signal results from engagement of T cell receptor (TCR) by its cognate antigen-major histocompatibility complex (MHC). The second signals involve a plethora of costimulation molecules. It appears, however, that CD4+CD25+ T cells do not need CD28 ligation for their function. CD4+CD25+ T cells from CD28-deficient mice exhibited equally potent suppressive activity in vitro as cells from wild-type animals [6]. A recent study also showed that inhibition of costimulatory signals did not inhibit the ability of fresh CD4+CD25+ T cells to suppress CD8+ T cell responses under the conditions in which activation of the responders was independent of costimulation [7]. These findings indicate that the activation of CD4+CD25+ T cells, to induce their suppressor function, is independent of the engagement of costimulation, hence highlighting the importance of the TCR-transduced signals in the function of CD4+CD25+ T cells. Limited information regarding TCR signaling in fresh human CD4+CD25+ T cells is available.

The proximal events following TCR engagement in conventional T cells include phosphorylation of tyrosine residues in immunoreceptor tyrosine-based activation motifs (ITAMs) in the TCR-CD3 complex by the src kinase p56lck. ζ-Associated protein-70 (ZAP-70) then binds to the phosphorylated CD3ζ complex, most notably to CD3ζ. This interaction of ZAP-70, with the phosphorylated ITAMs, allows interaction of p56lck with ZAP-70, thus leading to its phosphorylation and activation by p56lck [8, 9]. These initial events initiate the activation of a signal transduction cascade, resulting in activation of protein kinase C (PKC), mobilization of calcium, and activation of a

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Ras signaling cascade [8, 9]. This complex signaling system ultimately results in the up-regulation of genes that are critical for the expansion and effector functions of T cells.

The distinct outcome of CD4+/CD25+ T cells after TCR ligation suggests that intracellular signaling events may differ from those in CD4+/CD25− T cells. These signals may be critical for triggering the suppression mechanism and inducing the distinct behavior of CD4+ T cells through the activation of Ras signaling cascade [8, 9]. This complex signaling system ultimately results in the up-regulation of genes that are critical for the expansion and effector functions of T cells.

**MATERIALS AND METHODS**

**Culture media, reagents, and antibodies**

RPMI-1640 medium, supplemented with L-glutamine, penicillin/streptomycin, and 10% (v/v) human AB serum (Harlan Sera-Lab, Loughborough, UK), was used for all in vitro assays. For cell purification and washing steps, RPMI-1640 medium, supplemented with 2% fetal calf serum (FCS), was used. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma-Aldrich (UK). Anti-CD3-Tri-color (Clone S4.1), anti-CD4-allophycocyanin (APC; Clone S3.5), anti-CD25-phycocerythrin (Clone CD25-3G10), and anti-CD69-fluorescein isothiocyanate (FITC; Clone CH/4) were purchased from Caltag (San Francisco, CA). Antibodies to CD3/CD25/CD4/CD25 and CD4/CD25 were prepared from buffy coat samples (North London Blood Transfusion Center, UK) by first incubating the blood with RosetteSep human T cell enrichment cocktail (50 μl/ml blood, StemCell Technologies, Seattle, WA) at room temperature for 20 min, followed by density gradient centrifugation over Lymphoprep. The purified CD4+ T cells from the interlayer were then incubated with CD25 Dynal beads (Dynal Biotech, UK) for 30 min. CD4+/CD25− T cells were separated from CD4+/CD25+ T cells using a magnet. The unbound fraction contained the CD4+ T cells. To detach the CD4+CD25+ T cells from the Dynal beads, the cells bound to the beads were incubated with CD4/CD8 Detachbeads (Dynal Biotech) with shaking for 1 h at room temperature. The beads were removed from the isolated cells with a magnet. The purity of the cells was checked by flow cytometry.

**Preparation of human CD4+CD25− and CD4+CD25+ T cells**

CD4+ T cells were prepared from buffy coat samples (North London Blood Transfusion Center, UK) by first incubating the blood with RosetteSep human CD4+ T cell enrichment cocktail (50 μl/ml blood, StemCell Technologies, Seattle, WA) at room temperature for 20 min, followed by density gradient centrifugation over Lymphoprep. The purified CD4+ T cells from the interlayer were then incubated with CD25 Dynal beads (Dynal Biotech, UK) for 30 min. CD4+/CD25− T cells were separated from CD4+/CD25+ T cells using a magnet. The unbound fraction contained the CD4+CD25− T cells. To detach the CD4+CD25+ T cells from the Dynal beads, the cells bound to the beads were incubated with CD4/CD8 Detachbeads (Dynal Biotech) with shaking for 1 h at room temperature. The beads were removed from the isolated cells with a magnet. The purity of the cells was checked by flow cytometry.

**Generation of dendritic cells (DC)**

Peripheral blood mononuclear cells (PBMCs) were prepared from buffy coat preparation by density gradient centrifugation over Lymphoprep. Human monocyte-derived DC were generated by culturing adherent cells from PBMCs in the presence of granulocyte macrophage-colony stimulating factor (R&D Systems, UK) and interleukin (IL)-4 (R&D Systems). After 7 days, DC were harvested and were used in proliferation assays with CD4+CD25+ or CD4+CD25− T cells.

**Proliferation assay**

T cells (5 × 10^5 cells/well) were stimulated with γ-irradiated (30 Gray), allogeneic DC (1 × 10^5 cells/well), T cell expander beads (0.15 μl per well, Dynal Biotech), or PMA (1 ng/ml) plus ionomycin (1 μg/ml) in a 96-well plate. Proliferation was assessed by 3H-thymidine incorporation during the last 18 h of 5-day cultures when DC were used or 3-day cultures in the cases of T cell expander beads or PMA/ionomycin.

**Flow cytometric analysis**

All flow cytometry analysis was conducted on a Becton Dickinson FACSCalibur running CellQuest software (Becton Dickinson, UK). For surface staining, 5 × 10^6 cells were incubated with saturating concentrations of appropriate antibodies for 30 min at 4°C in the dark and then washed twice in cold fluorescein-activated cell sorter buffer [phosphate-buffered saline (PBS) with 1% (v/v) FCS and 0.01% (v/v) sodium azide] before analysis. For intracellular staining, the cells were first fixed with ice-cold 4% (v/v) paraformaldehyde for 20 min and then stained in a similar manner but with the presence of 0.1% (v/v) saponin (Sigma-Aldrich).

**T cell stimulation and Western blot analysis**

Purified CD4+CD25− and CD4+CD25+ T cells were incubated with 10 μg/ml anti-CD3 antibodies (OKT3) on ice for 5 min and then activated by cross-linking with 20 μg/ml goat anti-mouse antibodies (Dako, Denmark) at 37°C for 5 min. Cells incubated without anti-CD3 were used as nonactivated controls. For Western blot analyses and immunoprecipitation, 5 × 10^6 cells were lysed with ice-cold lysis buffer containing 1% (v/v) Triton-X 100, 1 mM Na3VO4, 1 mM EDTA, and protease inhibitor cocktail (Sigma-Aldrich) for 30 min. The lysate was cleared by centrifugation for 20 min at 13,000 g at 4°C. Protein concentration of the cell lysate was quantified using a Bio-Rad ( Hercules, CA) protein quantitation kit. Cell lysates (5 μg protein) were then separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes. To detect various signal molecules, membranes were blocked with 5% (w/v) skimmed milk for 1 h in PBS with 0.01% Tween 20 and then probed with the corresponding primary antibody. Bound antibody was revealed with HRP-conjugated anti-mouse or anti-rabbit antibodies using enhanced chemiluminescence (Amersham Biosciences). The intensity of the band was determined using the GelDoc-It system and LabWorks software (UVP Ltd., Cambridge, UK).

For immunoprecipitation, cell lysates corresponding to 5 × 10^6 cells were incubated with the antibody overnight at 4°C, followed by incubation with protein G-Sepharose beads (Sigma-Aldrich) for 3 h at 4°C. The beads were washed three times with ice-cold lysis buffer, and proteins were extracted by boiling the beads in SDS-PAGE sample buffer. To detect coimmunoprecipitation, the membranes were stripped for 10 min at room temperature with 0.2% (w/v) NaOH and reprobed with specific antibodies.

**Detection of cellular calcium flux**

To measure cellular calcium flux, CD4+CD25+ and CD4+CD25− T cells were resuspended at 10^5 cells/ml in Hanks’ balanced saline solution containing 1 mM calcium and 1% (v/v) FCS with 4 μg/ml Fluor-3 AM (Molecular Probes, Eugene, OR). After 30 min incubation at room temperature, the cells were washed with Hanks’ calcium-free medium followed by incubation with 10 μg/ml anti-CD3 antibody on ice for 10 min. An increase in intracellular calcium was recorded in real time for 600 s on live gated cells using a FACSCalibur flow cytometer (Becton Dickinson) immediately after addition of cross-linking goat anti-mouse Ig (20 μg/ml). The data were analyzed using FlowJo software (Treestar, San Carlos, CA).

**Immunofluorescence microscopy**

Purified CD4+CD25+ and CD4+CD25− T cells were activated with T cell expander beads overnight at 37°C. Cells were harvested and fixed with 4% paraformaldehyde for 4°C for 20 min. Cells were washed and stained for actin with Texas Red-phalloidin (Sigma-Aldrich) at 37°C for 30 min. Specimens were analyzed by confocal microscopy (Zeiss LSM 10 confocal with LSM Analysis software, Zeiss, Thornwood, NY).
RESULTS

**CD4+CD25+** T cells were anergic and suppressive upon TCR activation, but the effects were reversed by PMA/ionomycin

Human CD4+CD25− and CD4+CD25+ T cells were purified from buffy coats (over 90% purity) and analyzed for their ability to respond to TCR ligation. Using T cell expander beads or allogeneic DC as stimulators, CD4+CD25− T cells were anergic upon TCR engagement and suppressed the proliferative response of the CD4+CD25− T cells (Fig. 1, A and B). To bypass TCR/CD3 activation, CD4+CD25− and CD4+CD25+ T cells were also stimulated with PMA and ionomycin. Under this condition, suppression and anergy of CD4+CD25+ T cells were reversed, suggesting that the downstream signaling events leading to proliferation are still intact, and the proximal TCR signaling of these cells may be different (Fig. 1C).

**CD4+CD25+** T cells are defective in the early steps in TCR signal transduction

As an initial assessment of TCR signaling, we analyzed the two major signaling pathways, the calcium-calcineurin and Ras signaling cascades, which are induced by PMA and ionomycin. The ability of CD4+CD25+ and CD4+CD25− T cells to mobilize intracellular calcium stores and to phosphorylate ERK after TCR ligation was analyzed. Fluo-3 AM-loaded, purified CD4+CD25− T cells and CD4+CD25− T cells were stimulated by anti-CD3 cross-linking. Increases in intracellular calcium were recorded in real time for 600 s on live gated cells. CD4+CD25− T cells responded well to anti-CD3 stimulation (peak intensity = 4.3), whereas the CD4+CD25+ T cells were refractory (peak intensity = 2.4; Fig. 2A).

Activation of ERK was assessed using a phospho-specific antibody, which recognizes ERK-1 and ERK-2. PMA/ionomycin stimulation and TCR ligation caused a marked increase in ERK-1 and ERK-2 phosphorylation in CD4+CD25− and CD4+CD25+ T cells. PMA/ionomycin induced a comparable level of phosphorylation in both populations. However, the increase as a result of TCR ligation detected in CD4+CD25− T cells was ~40% of that of CD4+CD25− T cells, as judged by densitometric analysis (Fig. 2B). Efficient induction of CD69 has been shown to be dependent on the Ras-signaling pathway [11]. Therefore, we also analyzed CD69 up-regulation after overnight TCR stimulation. Only 16% of CD4+CD25− T cells up-regulated CD69, and 47% of CD4+CD25− T cells became CD69-positive after overnight stimulation with T cell expander beads (Fig. 2C). The lower level of CD69 also reflected the defect in activation of Ras signaling, corresponding to the reduced levels of ERK activation.

A marked reduction in calcium flux and ERK phosphorylation suggested a defect in a common upstream signaling pathway. One of the key initiating events in T cell activation is phosphorylation of ITAMs in the TCR-CD3 complex. This, in turn, results in ZAP-70 recruitment. Both of these events were analyzed following TCR ligation. Immunoprecipitation of the CD3ζ chain in CD4+CD25+ T cells showed lower CD3ζ chain phosphorylation than CD4+CD25− T cells (Fig. 3A). Also, coimmunoprecipitation of ZAP-70 with the CD3ζ chain was detected only in activated CD4+CD25− T cells. In addition, freshly isolated CD4+CD25+ T cells...
showed lower surface expression of CD3ε (Fig. 3B) and TCR (data not shown).

SLP-76 acts as a central adaptor in TCR signaling and is a key substrate of activated ZAP-70 [12]. Impairment of CD3ζ chain phosphorylation is generally accompanied by a defect in ZAP-70 activation. Phosphorylation of SLP-76 after anti-CD3 cross-linking in CD4+CD25+ T cells and CD4+CD25− T cells was compared. Immunoprecipitation of SLP-76 showed that only activated CD4+CD25− T cells had a detectable level of tyrosine phosphorylation of SLP-76 (Fig. 4). Given that phosphorylated SLP-76 binds to VAV, we further confirmed its phosphorylation status following VAV coimmunoprecipitation. Figure 4 shows VAV coimmunoprecipitation with SLP-76 only in activated CD4+CD25− T cells but not CD4+CD25+ T cells.

Alteration in early TCR signaling in CD4+CD25+ T cells caused defective actin polymerization

One major consequence of SLP-76 phosphorylation is the recruitment of VAV, which has guanine nucleotide exchange factor activity for Rho and Rac1—proteins that regulate actin remodeling [9]—required for sustained TCR signaling [13]. Having observed a defect in the phosphorylation of SLP-76, we examined the ability of CD4+CD25+ T cells to form actin caps after TCR ligation. Following activation, actin cap formation was visualized by phalloidin staining in CD4+CD25− T cells. This activation-dependent change in F-actin morphology was virtually absent from CD4+CD25+ T cells following stimulation (Fig. 5), confirming a lack of VAV recruitment and initial upstream TCR signaling.

DISCUSSION

CD4+CD25+ T cells are potent, immunoregulatory cells, which not only suppress T cell proliferation in vitro but also have the capacity to suppress immune responses to autoimmunity and alloantigens in vivo [1–3]. In vitro studies of CD4+CD25+ T cells have demonstrated that these cells are hyporesponsive. A closed chromatin configuration at the IL-2 promoter has been suggested to account for their defect in IL-2 production leading to hyporesponsiveness [14]. Neither TCR nor IL-2 receptor (IL-2R) signaling alone can reverse their hypoproliferative phenotype, but CD4+CD25+ T cells become proliferative when the signals are combined [15–17]. TCR signaling has been suggested to restore IL-2R signaling;
thus, IL-2 induced proliferation [17]. TCR signals are not only required for CD4+CD25+ T cell proliferation, they are also important for their suppressive function. A number of reports have shown that TCR activation is required for the cells to acquire suppressive function [4, 5]. Despite the importance of TCR signals in CD4+CD25+ T cells, little is known about the signals that regulate CD4+CD25+ T cell proliferation and suppression. It could be an active or a passive process, involving blockade, modulation, or alteration in signal transduction pathways. The observations reported here suggest that TCR signaling events in human CD4+CD25+ T cells are markedly dampened as compared with those seen in CD4+CD25− T cells.

CD4+CD25+ T cells are anergic and suppressive upon TCR ligation. However, this is reversed following PMA/ionomycin stimulation. PMA, a PKC activator, activates Ras by suppressing the activity of guanosine triphosphatase-activating protein RasGAP [18]. When PMA is used in conjunction with ionomycin, a calcium ionophore, signaling pathways downstream of Ras and PKC can be activated without activation of

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**Fig. 3.** TCR ligation in CD4+CD25+ T cells resulted in lower CD3ζ chain phosphorylation and recruitment of ZAP-70, accompanied by lower CD3ε surface expression. (A) Purified T cells were activated by CD3 cross-linking for 5 min at 37°C. Cell lysates were immunoprecipitated (IP) with anti-CD3ζ antibody with protein G-Sepharose beads. The proteins were separated by 4–16% gradient SDS-PAGE and Western blotted. The blot was first probed with antiphosphotyrosine antibody (4G10) to detect CD3ζ chain phosphorylation and then stripped and reprobed with anti-CD3ζ chain and anti-ZAP-70 antibodies. O.D., optical density; vertical stripes, resting control; dots, activated CD25+; diagonal stripes, activated CD25−. (B) The surface expression of CD3ε by freshly isolated cells was detected before TCR ligation using anti-CD3-TC.

**Fig. 4.** TCR ligation of CD4+CD25+ T cells resulted in lower SLP-76 phosphorylation and recruitment of VAV. Purified T cells were activated by CD3 cross-linking for 5 min at 37°C. Cell lysates were immunoprecipitated with anti-SLP-76 antibodies followed by protein G-Sepharose beads. The proteins were separated by 10% SDS-PAGE. The blot was first probed with antiphosphotyrosine antibody (4G10) to detect SLP-76 phosphorylation and then stripped and reprobed with anti-SLP-76 and anti-VAV antibodies.
proximal TCR-triggered signaling events. Consistent with previous studies of mouse, human CD4+CD25+ T cells proliferate upon PMA/ionomycin stimulation, demonstrating that downstream signaling pathways leading to T cell proliferation in these cells are intact [14]. The absence of suppression may be a result of the fact that induction of suppressive function of CD4+CD25+ T cell requires activation of more upstream events or the resistance of CD4+CD25− T cells to be suppressed under PMA/ionomycin stimulation.

Examining the signaling events, which were affected directly by PMA and ionomycin—calcium flux and ERK activation—we observed that there were reductions in calcium flux and activation of ERK upon TCR ligation in CD4+CD25+ T cells but not with PMA/ionomycin stimulation (Fig. 2). Down-modulation of both signaling pathways indicated that there could be an alteration of the common upstream events in the proximal TCR signaling. The earliest detectable event upon TCR ligation is the activation of the src family tyrosine kinase, p56lck, which subsequently phosphorylates the ITAMs in the intracellular domain of CD3ε and CD3ζ chains. Phosphorylation of tyrosine residues in the CD3ζ chain establishes a binding site for ZAP-70, which can be tyrosine-phosphorylated by p56lck, leading to an up-regulation of its activity. Subsequently, ZAP-70 phosphorylates other downstream substrates, such as SLP-76, linker for activation of T cells, and others [8, 9]. We have shown that the alteration of TCR signaling in CD4+CD25+ T cells occurred at the earliest step. TCR ligation resulted in a reduction in CD3ζ chain phosphorylation in CD4+CD25+ T cells as compared with CD4+CD25− T cells. It was accompanied with poor recruitment of ZAP-70 to the CD3ζ chain, as shown by coimmunoprecipitation with CD3ζ (Fig. 3A). A reduction in ZAP-70 activation was observed, as shown by lower phosphorylation of its substrate, SLP-76 (Fig. 4). Studies using CD4+CD25+ T cell lines have shown that they share many biochemical characteristics with anergic T cells, including increased expression of p27kip1, defective cell cycle progression, and defective Ras signaling [15, 19]. Here, we found that ERK phosphorylation, in freshly isolated human CD4+CD25+ T cells, is defective. In addition to that, we found low ZAP-70 activation, but unlike anergic cells, fresh human CD4+CD25+ T cells showed a markedly reduced calcium flux [19]. In this respect, CD4+CD25+ T cells are a distinct type of anergic T cell. The specific alterations in TCR signaling observed in the CD4+CD25+ T cells can be used as a biochemical marker to distinguish them from other regulatory cells.

Although proximal TCR signaling in CD4+CD25+ T cells is impaired, these events are sufficient to induce down-regulation of phosphoinositide-3-phosphatase (PTEN) expression in mouse CD4+CD25+ T cells [17]. It has been suggested that PTEN down-regulation after TCR stimulation can restore II-2-mediated phosphatidylinositol 3-kinase-dependent signaling and proliferative capacity of CD4+CD25+ T cells [17]. Although we did not find PTEN down-regulation in our system (supplementary data), it is possible that PTEN activity could be regulated in other ways in humans [20].

The altered TCR signals in CD4+CD25+ T cells affected actin cap formation upon TCR ligation (Fig. 5). The actin cytoskeleton plays an important role in T cell activation. Upon stimulation, a number of surface receptors and signaling molecules, such as CD4, TCR, CD3ζ, and p56lck, are recruited to the actin cytoskeleton [21]. It guides the formation of the immunological synapse at the interface between T cells and antigen presenting cells (APCs). Also, actin-dependent clustering may increase effective concentrations of signaling molecules and enable signal perpetuation beyond expiration of initial stimuli [13]. Defective actin cap formation may lead to a defect in actin remodeling in CD4+CD25+ T cells. The perturbation of immunological synapse formation and subsequent lack of sustained p56lck activation could further down-modulate proximal TCR signaling.

There are several possible explanations for the down-modulation of TCR signals in CD4+CD25+ T cells. First, a growing body of evidence supports the idea that CD4+CD25+ T cells are reactive to self-antigen [4, 22, 23]. Therefore, their TCRs could be engaged continuously in vivo. This engagement can lead to down-modulation of cell surface expression of the TCR-CD3 complex, thus, T cell unresponsiveness [24]. The lower CD3ζ surface expression in the CD4+CD25+ T cells observed (Fig. 3B) could be a result of continuous TCR ligation by self-antigens in vivo. Maximal TCR down-regulation has been reported by 5 h poststimulus, but it can be maintained for up to several days poststimulation [25]. Interfering TCR down-modulation has been shown to enhance cell signaling [26]. Hence, it is possible that down-modulation of cell surface receptor level may limit antigenic signaling in T cell activation.

A second possibility is that CD4+CD25+ T cells with altered TCR signal transduction may be selected during their thymic development. Commitment to the CD4+CD25+ T cell lineage would require an especially fine balance between deletion and an intermediate level of signaling, resulting in the generation of a conventional effector T cell. During ontogeny of the immune system, T cells expressing high-affinity TCRs with a strong signal for self-antigen undergo clonal deletion in the thymus against a background of positive selection to MHC and self-antigen. However, the selection of CD4+CD25+ T cells has been shown to depend on high TCR affinity for selecting peptides. It seems to be contrasted with the concept of negative selection [27, 28]. It is not clear how high-affinity interactions of CD4+CD25+ T cells with self-antigen can escape the negative selection. The altered TCR signal generated by

Fig. 5. TCR ligation of CD4+CD25+ T cells did not induce actin cap formation. Purified T cells were activated with T cell expander beads for 30 min at 37°C. The cells were fixed and stained with Texas Red-phalloidin. Actin cap formation (as indicated by the arrow) was visualized by confocal microscopy.
CD4+CD25+ T cells after TCR engagement observed in our study may provide an explanation.

A third alternative is created by up-regulation of several molecules including cytotoxic T lymphocyte antigen-4 (CTLA-4), FOXP3, program death-1, and glucocorticoid-induced TNF receptor-related protein in CD4+CD25+ T cells [29, 30]. Some of these molecules have been implicated in regulation of TCR signals. For example, CTLA-4 has been reported to suppress proximal TCR signaling [31, 32]. Thus, molecules up-regulated in CD4+CD25+ T cells may play a role in modulation of their TCR signal.

In summary, we have demonstrated that there are alterations in the proximal TCR signaling events in human CD4+CD25+ T cells. The alteration occurred at the early stages of TCR signal transduction, with specific reduction of CD3 epsilon chain and SLP-76 phosphorylation, thus leading to failure of activation of downstream targets, such as calcium influx and ERK activation. The altered signals also resulted in an alteration of cytoskeletal remodeling in CD4+CD25+ T cells. These alterations could be critical for the distinct behavior of these cells.

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