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Anergic CD4 T Cells Form Mature Immunological Synapses with Enhanced Accumulation of c-Cbl and Cbl-b

Melissa Doherty,*† Douglas G. Osborne,* Diana L. Browning, † David C. Parker, ‡ and Scott A. Wetzel*†

CD4+ T cell recognition of MHC:peptide complexes in the context of a costimulatory signal results in the large-scale redistribution of molecules to the T cell–APC interface to form a mature immunological synapse. The immunological synapse is the location of sustained TCR signaling and delivery of a subset of effector functions. T cells activated in the absence of costimulation are rendered anergic and are hyporesponsive when presented with Ag in the presence of optimal costimulation. Several previous studies have looked at aspects of immunological synapses formed by anergic T cells, but it remains unclear whether there are differences in the formation or composition of anergic immunological synapses. In this study, we anergized primary murine CD4+ T cells by incubation of costimulation-deficient, transfected fibroblast APCs. Using a combination of TCR, MHC:peptide, and ICAM-1 staining, we found that anergic T cells make mature immunological synapses with characteristic central and peripheral supramolecular activation cluster domains that were indistinguishable from control synapses. There were small increases in total phosphotyrosine at the anergic synapse along with significant decreases in phosphorylated ERK 1/2 accumulation. Most striking, there was specific accumulation of c-Cbl and Cbl-b to the anergic synapses. Cbl-b, previously shown to be essential in anergy induction, was found in both the central and the peripheral supramolecular activation clusters of the anergic synapse. This Cbl-b (and c-Cbl) accumulation at the anergic synapse may play an important role in anergy maintenance, induction, or both. The Journal of Immunology, 2010, 184: 3598–3608.

Antigen recognition by CD4+ T cells triggers the large-scale spatial redistribution of molecules to the T cell–APC interface to form a mature immunological synapse (1, 2). The mature immunological synapse is characterized by the segregation of molecules into central and peripheral supramolecular activation clusters (cSMACs and pSMACs, respectively) (2). The prototypical mature immunological synapse has MHC:peptide, TCR, and protein kinase Cθ (PKCθ) accumulated in the cSMAC and molecules including ICAM-1 and LFA-1 accumulated in the pSMAC (1–3). Since its initial description more than a decade ago, there has been intense interest in the functions of this structure. The immunological synapse is the location of sustained signaling and TCR downmodulation (4–7). It also serves as the location of directional secretion for a subset of effector cytokines (8) and secretion of cytolytic granules by CD8+ T cells (9).

We, and others, have shown that costimulation enhances the formation of the mature immunological synapse (10–12). In the absence of costimulation, T cells are rendered anergic (13, 14), a hyporesponsive state characterized by alterations in intracellular signaling and establishment of an “anergy program” at the transcriptional level (14–18). One of the molecules expressed at significantly higher levels in anergic T cells is the E3 ubiquitin ligase Cbl-b (19–21). Cbl-b has been implicated in controlling the establishment or maintenance, or both, of the anergic phenotype (16, 20, 22). Cbl-b is expressed predominantly in mature peripheral T cells and is a negative regulator of TCR signaling (23). The closely related c-Cbl is found predominantly in thymocytes (24) and functions in the regulation of positive selection (25). Cbl-b (and c-Cbl) accumulation at the anergic synapse may play an important role in anergy maintenance, induction, or both.
alterations in intracellular signaling, the transcriptional program, and effector functions, we hypothesized that the immunological synapses formed by anergic T cells would be significantly different from control synapses. Several previous studies have examined aspects of the anergic immunological synapse (19, 38–41) and have shown that there is reduced recruitment of linker of activated T cells (LATs) (41) and lipid rafts, as shown by cholestery toxin B staining (39), and that on anergic CD8+ T cells there is reduced CD45, Lck, and CD8 accumulation (40). However, only two studies have examined the spatial distribution of molecules at the anergic synapse, and their results are contradictory. Carlin et al. (38) found CD3 distributed in an arc or ring structure at the interface, whereas Heissmeyer et al. (19) found normal MHC:peptide recruitment to the mature anergic synapse at early time points (<22 min). However, Heissmeyer et al. (19) did report breakdown of the pSMAC ring at time points >22 min. Clearly, much remains unknown about the immunological synapses formed by anergic T cells.

In this report, we have examined the immunological synapses formed by anergic CD4+ T cells and compared their morphology and molecular constituents to those of normal, rested T cell blasts. We show that anergic T cells form mature immunological synapses with the characteristic cSMAC and pSMAC domains. The amount and spatial distribution of accumulated MHC:peptide complexes at the anergic synapse do not differ significantly from those of the control synapse. However, when examining TCR signaling-associated molecules, we saw a slight increase in phosphorylated tyrosine (pTyr) and a significant reduction in phosphorylated ERK (pERK) in the anergic synapse. F-actin accumulation was slightly reduced, but the spatial distribution was indistinguishable from that of controls. Most significantly, we observed enhanced Cbl-b and c-Cbl recruitment to the T cell–APC interface. Although these known negative regulators of TCR signaling were enhanced in the pSMACs of both control and anergic cells, the accumulation was significantly higher for the anergic cells. There was also accumulation of Cbl-b to the cSMAC of the anergic but not control T cells. Thus, anergic CD4+ T cells do make mature immunological synapses but with altered molecular constituents.

Materials and Methods

Animals

Heterozygous AD10 TCR transgenic mice (Vβ3+), specific for pigeon cytochrome c fragment 88–104 (42) and reactive against moth cytochrome c (New Hope CA). Anti-CD4, anti-CD8, and anti-CD45 (RM4-5) were purchased from BioLegend (San Jose, CA). Anti-CD4 (RM4-5) was purchased from R&D Systems (Minneapolis, MN). Anti-CD8 (R3-3.5) was purchased from BD Biosciences (San Jose, CA). Anti-CD4 (RM4-5) was purchased from BioLegend (San Diego, CA). A polyclonal rabbit Ab to gene related to anergy in lymphocytes (GRAIL) (44) was a kind gift of Dr. Garrison Fathman (Stanford University, Palo Alto, CA).

Peptides

MCC88–103 and HbD4.76 were purchased from New England Peptide (Gardiner, MA) and resuspended at 500 μM in PBS. They were diluted in complete RPMI 1640 for use.

APCs

Transfected Ltk− fibroblasts were used as APCs to induce anergy and in subsequent imaging and flow cytometry experiments. MCC:GFP fibroblasts expressing GFP-tagged Eμ-Cμ β-chain with covalent antigenic MHC peptide have been described previously (12). The LMCC cell line expressing an MHC-I-Ek β-chain covalent construct with three repeats of the DNA gyrB domain on the cytoplasmic tail have also been described previously (45). The levels of Eμ-MHC on the LMCC and MCC:GFP cells are very similar, but LMCC cells express very low levels of CD80 and CD40 (12, 46). Cells were maintained in DMEM (Sigma-Aldrich, St. Louis, MO) containing 10% FBS (Atlanta Biologicals, Atlanta, GA) and supplemented with 1 mM l-glutamine, 100 mg/ml sodium pyruvate, 50 μM 2-ME, essential and nonessential amino acids, 100 U/ml penicillin G, 100 U/ml streptomycin, and 50 μg/ml gentamycin (Sigma-Aldrich).

The Eμ-MHC CH27 B cell lymphoblast cell line, a gift of Brian Evavold (Emory University, Atlanta, GA), was used to present Ag in proliferation assays. These cells were maintained in RPMI 1640 (Sigma-Aldrich) containing 10% FBS (Atlanta Biologicals) and supplemented with 1 mM l-glutamine, 100 mg/ml sodium pyruvate, 50 μM 2-ME, essential and nonessential amino acids, 100 U/ml penicillin G, 100 U/ml streptomycin, and 50 μg/ml gentamycin.

CD4+ T cell generation and energy induction

AD10 spleens were harvested, and single-cell suspensions were prepared by gentle grinding between sterile glass slides. After hypotonic lysis of RBCs, lymphocytes were enriched by Ficoll–Hypaque. Lymphocytes were either (a) fixed with 4% paraformaldehyde (PFA) or (b) permeabilized in 0.5% glutaraldehyde in PBS (Sigma-Aldrich) and incubated for 45 min at room temperature. Lymphocytes were resuspended at 5×10^6 cells/ml in complete RPMI 1640, and 2.5 μM MCC peptide was added. Day 3 T cell blasts cells were harvested from the in vitro culture, and live cells were enriched by density centrifugation on Percoll solution.

Measurement of T cell proliferation

Proliferation of the AD10 T cells in response to MCC88–103 peptide-pulsed CH27 cells was measured by a standard 7 h [3H]thymidine incorporation assay. A total of 2.5×10^4 control or anergicized AD10 T cells were incubated with 5×10^5 mitomycin C-treated CH27 cells pulsed with varying amounts of MCC88–103 peptide. Cultures were pulsed with 1 μCi [3H] thymidine (2 Ci/mmol sp. act.) during the last 12 h of a 72 h assay. Cells were harvested, and radioactivity was measured using a Packard TopCounter (Packard Instruments, Waltham, MA).

T cell activation by flow cytometry

A total of 2.5×10^6 T cells and 1×10^6 MCC:GFP cells per well in a six-well plate were incubated overnight (12–18 h) in 3 ml total volume of complete RPMI 1640. T cells were recovered from the wells and stained for CD4, Vβ3, and CD69 for 30 min at 4°C in FACS buffer (PBS 2% BSA fraction V 0.1% NaN3). After three washes, cells were stained for 20 min with secondary reagents in FACS buffer. After three additional washes, cells were examined using a FACSARia (BD Biosciences), and data were analyzed using FlowJo 8 (Tree Star, Ashland, OR).

Fixed cell microscopy

Imaging experiments were carried out in #1.5 LabTek II eight-chambered coverslips (Nunc, Rochester, NY). One day before use, 2.5×10^6 MCC:GFP cells were added per well and incubated overnight at 37°C. After addition of 10^5 T cells, dishes were spun briefly to initiate contact between T cells and APCs and subsequently incubated for 30 min at 37°C. Cells were fixed by addition of ice-cold fixative (4% paraformaldehyde and 0.5% glutaraldehyde in PBS) and incubated for 45 min at room temperature in the dark followed by permeabilization with 0.2% Triton X-100 in
PBS for 10 min. Cultures were stained with primary Abs at concentrations of 10 μg/ml in PBS for 2 h at room temperature in a humidified chamber. After three PBS washes, cells were incubated with secondary Abs at concentrations of 5 μg/ml or phalloidin (1:500 dilution in PBS) for 2 h at room temperature. After three additional PBS washes, SlowFade Gold antifade reagent (Molecular Probes) was added to the wells. Samples were stored at 4°C and protected from light until imaged.

T cell–APC conjugates to be imaged were chosen based upon their characteristic morphology in differential interference contrast (DIC) of T cells in tight contact with and flattened against an APC. A stack of 50–90 fluorescent images spaced 0.2 μm apart in the z-axis was obtained with a 60×, 1.4 NA, oil immersion lens on the Applied Precision (API) DeltaVisionRT image restoration microscopy system (Issaquah, WA). Deconvolution was performed using an iterative, constrained algorithm module in the API SoftWorx software package. Three-dimensional (3D) reconstructions and further image analysis were performed using the API SoftWorx software package. Colocalization was analyzed using ImageJ (47) and the JACoP plugin (48) to calculate the Pearson’s correlation coefficient.

**Live cell microscopy**

For live cell microscopy, 2.5 × 10⁴ APCs were seeded into 81.5 LabTek II eight-chambered coverslips 1 d prior to the experiment. Dishes were fitted onto the stage of the API DeltaVisionRT image restoration system. Temperature was maintained at 37°C for the duration of the imaging by the Weather Station environmental chamber. After 1 × 10⁴ AD10 T cells were added to a well, alternating green fluorescent (528 nm) and DIC images were taken every 8–12 s for 60 min using a 60×, 1.4 NA lens. Image analysis was performed using the API SoftWorx software package along with ImageJ (47). Live-cell experiments were performed in complete RPMI 1640 culture medium buffered with 10 mM MOPS and free of phenol red and bicarbonate.

**Statistical analysis and graphing**

Statistical analysis (ANOVA and Student t test) and graphing were performed using Prism 4 (GraphPad Software, La Jolla, CA). Significance was defined as p ≥ 0.05.

**Results**

AD10 CD4⁺ T cells are rendered anergic by 24 h incubation on transfected fibroblast APCs that express very low levels of CD80

The present study was designed to determine whether in vitro anergized CD4⁺ T cells form mature immunological synapses and, if so, to characterize these synapses. Anergy was induced by a 24 h incubation of day 4 T cell blasts on CD80 low LMCC fibroblast APCs as described previously (45). To confirm anergy induction, the T cells were rested for 6 d after recovery from the coculture with the LMCC cells before restimulation with CH27 B cells pulsed with MCC88-103 antigenic peptide. As seen in Fig. 1A, the proliferative capacity of the T cells previously incubated with the LMCC fibroblast APCs was significantly reduced compared with that of the control cells. Addition of 20 U/ml recombinant murine IL-2 during the 3 d proliferation assay rescued the proliferative capacity of the T cells precultivated on the LMCC cells. These results confirm that incubation with the LMCC fibroblast APCs induces anergy in responding Ag-specific CD4⁺ T cells.

The ability of the anergized CD4⁺ T cells to respond to Ag was further characterized after overnight incubation on MCC:GFP fibroblasts (12). These cells express high levels of endogenous CD80 and have been transfected with ICAM-1, I-E^k α-chain, and a GFP-tagged I-E^k β-chain with covalently attached MCC88-103 antigenic peptide (12). To assess the Ag responsiveness of the control and anergic T cells, we measured the surface expression of the TCR.

Both control and anergized T cells clearly downmodulated their TCR after overnight stimulation by MCC:GFP cells (Fig. 1B), showing that both were Ag responsive. Of note, the extent of TCR downmodulation on stimulated cells was significantly higher for anergic T cells than that for control T cells, even though these cells had significantly reduced proliferation in response to Ag. The level of CD69 expression, an early activation marker, was increased on both control and anergic T cells after stimulation with the MCC:GFP cells (Fig. 1C). However, when compared with unstimulated T cells (filled histograms), the increase in CD69 expression on Ag-stimulated anergic cells was significantly less than that seen on the control cells. The phenotype and proliferation of T cells anergized by 24 h incubation on the LMCC fibroblasts are identical to those of T cells anergized by 24 h incubation on anti-TCRβ–coated plates (data not shown). Taken together, the results in Fig. 1 show that 24 h coculture with LMCC fibroblasts induces anergy in Ag-specific AD10 T Cell blasts. These anergized T cells remain Ag responsive, with the level of TCR downmodulation significantly greater and CD69 upregulation significantly lower than those in control T cells following exposure to optimal stimulation.

**Anergic T cells form stable T cell–APC conjugates at a reduced frequency**

The results in Fig. 1 show that CD4⁺ AD10 T cells were rendered anergic by coculture with the CD80low LMCC fibroblast APCs and retain the ability to interact with APCs. Contact between CD4⁺ T cells and APCs initiates large-scale cytoskeletal and morphological changes within the T cells, leading to conjugate formation and an increase in the T cell–APC contact area (49–51). The ability of anergized T cells to form stable conjugates with APCs is unresolved. There have been studies showing no differences compared...
with control T cells (39, 41), whereas others show significant reductions in conjugate formation by anergic T cells (38, 40). The formation of T cell–APC conjugates is a prerequisite for the formation of mature immunological synapses; therefore it was essential to determine whether the in vitro anergized T cells formed stable conjugates with APCs. DiI-labeled T cells were mixed with MCC:GFP fibroblast APCs, and conjugate formation was determined after 30 min by flow cytometry.

As seen in Fig. 2A, after a 30 min incubation, control T cells form stable conjugates with the MCC:GFP fibroblast APCs. The anergic T cells also form stable conjugates, but at a lower frequency than that seen with the control T cells (12.7 versus 7.27%, respectively). Although the T cell–APC conjugate population formed by the anergic T cells was not as distinct as that of control cells, the frequency of anergic conjugates was still significantly higher than that seen with the non-specific I-Ek:HLA.A-reactive 3.12 T cells (4.11%). Although the percentage of conjugates varied slightly from experiment to experiment (control range was 6.17–16.4% and anergic range was 4.4–11.9%), the difference between the three groups was consistent within each experiment. The frequency of stable control T cell–APC conjugates was 69.3 ± 8.45% (mean ± SEM) higher than the frequency of stable anergic T cell conjugates and 188.8% ± 24.1% higher than that for the non-specific 3.12 T cells.

Kinetics of immunological synapse formation by anergic T cells are similar to those of control T cells

The data in Fig. 2A showed that both control and anergic T cells have the ability to form stable conjugates, albeit with different frequencies. To determine whether anergic CD4+ T cells have the ability to form mature immunological synapses, we assessed the redistribution of the GFP-tagged MHC:peptide complexes and monitored T cell morphology during T cell–APC interactions by live-cell microscopy. Using this system, we previously showed that interaction with Ag-specific T cells leads to large-scale T cell morphological changes and formation of small MHC:peptide clusters that coalesce to form an immunological synapse (12). In live-cell imaging experiments, we have defined a “mature” immunological synapse as a GFP cluster at the interface of a T cell and APC that has reached maximal size and fluorescence intensity (12).

As seen in Fig. 2B, the interaction of both control and anergic T cells with the MCC:GFP APC results in tight adherence and flattening against the APC with a dramatic increase in the contact area over a period of several minutes. After observing at least 125 individual T cell–APC interactions for both control and anergic T cells over five separate experiments, we found that the characteristic morphological changes occurring as a consequence of Ag recognition were indistinguishable between control and anergic T cells.

We also examined the kinetics of immunological synapse formation for control and anergic T cells during the live-cell experiments by calculating the time between the initial T cell contact with an APC and the formation of the mature immunological synapse. For the control cells, the mean time between contact and mature immunological synapse formation was 394 ± 55.9 s (mean ± SEM) with a range of 180–1159 s. For the anergic cells, the mean time between contact and mature immunological synapse formation was slightly longer at 434 ± 92 s with a range of 244–1447 s. As with the morphological data, there was no significant difference in the kinetics of mature immunological synapse formation between control and anergic T cells.

Anergic T cells form mature immunological synapses with prototypical cSMAC and pSMAC domains

To confirm that the structures observed by live-cell microscopy were indeed immunological synapses, we performed 3D microscopy using fixed and Ab-stained T cell–APC conjugates. A representative set of images from at least 200 control and anergic synapses over five separate experiments is shown in Fig. 2C. These images show specific accumulation of both the GFP-tagged MHC:peptide and the TCR at the interface of the control T cells and the APCs. The intensity of TCR staining at the interface of both control and anergic T cells was at least 3-fold above that of other areas on the same T cell. The TCR and GFP-tagged MHC:peptide colocalized at the T cell–APC interface as seen by the yellow region at the interface in the merged image. The second T cell attached to the left side of the APC forms a similar TCR–MHC colocalized region in a separate optical section (data not shown). As with the live-cell data, from an edge-on view, the colocalization of TCR and GFP-tagged MHC:peptide at the interface of the anergic T cells and the control T cells was virtually indistinguishable (Fig. 2C).

To confirm the TCR–MHC:peptide colocalization and to examine the spatial distribution of the MHC and TCR molecules at the T cell–APC interface, we generated 3D rotations of the interface from the optical sectioning data. For both control and anergic T cell interfaces, there was very clear colocalization of the MHC:peptide and the TCR at the T cell–APC interface (yellow regions in Fig. 2D). The Pearson’s correlation coefficients for areas of TCR intensity 3-fold or more over background localizing with areas of MHC–GFP 2-fold or more than background were 0.87 for control cells and 0.92 for the anergic cells. Thus, there was significant colocalization of the TCR and MHC:peptide at the T cell–APC interface for both control and anergic T cells, and there were no significant differences between the two groups.

The TCR and MHC:peptide are both constituents of the cSMAC in the mature immunological synapse (2). To confirm that the accumulated material at the T cell–APC interface was part of a traditional, spatially segregated mature immunological synapse, we next examined components of the pSMAC, ICAM-1 and its ligand LFA-1. The images in Fig. 2E are representative of >200 T cell–APC conjugates imaged for both control and anergic T cells over five separate experiments. For both control and anergic T cells, there was central accumulation of the GFP-tagged MHC:peptide with a ring of ICAM-1 at least 2-fold above background surrounding the central GFP spot. Similar distributions were observed with LFA-1 staining (data not shown). This arrangement is characteristic of the cSMAC and pSMAC spatial distribution seen in mature immunological synapses (1, 2).

Taken together, the data from Fig. 2 show that anergic T cells can form stable conjugates with APCs, and upon the interaction with APCs, there is redistribution of specific MHC:peptide complexes to the T cell–APC interface with similar kinetics to control T cells. Further, this interaction leads to spatial segregation of TCR and MHC:peptide to a central region at the interface (the cSMAC) by a ring of ICAM-1 (the pSMAC). These are prototypical features of mature immunological synapses, and their appearance at the anergic T cell–APC interface strongly supports the idea that anergic T cells are capable of forming mature immunological synapses.

Spatial distribution and the amount of accumulated MHC:peptide at the anergic immunological synapse are similar to those of controls

Having shown that anergic T cells form immunological synapses with the characteristic cSMAC and pSMAC molecular segregation patterns, we began characterizing the synapses further to determine if there were qualitative or quantitative differences in the accumulation or distribution, or both, of MHC:peptide complexes between anergic and control synapses.

To calculate the area of the T cell–APC interface with accumulated GFP-tagged MHC:peptide, we generated en face views of
FIGURE 2. Anergized T cells form T cell–APC conjugates with reduced efficiency but form mature immunological synapses with normal kinetics. A, Dil-stained control (left), anergic (center), or nonspecific (right) T cells were incubated for 30 min with APCs transfected with GFP-tagged MHC:peptide complexes. Conjugate formation was examined by flow cytometry. The numbers represent the percentages of T cells found in conjugates with APC. This is representative of four separate experiments. B, Live-cell imaging of immunological synapse formation by control and anergic T cells. Orange arrows indicate the T cell–APC interface where the immune synapse is forming. Point of initial contact is defined as time 0, and the mature immunological synapse is found in the right column at the indicated time point. At least 125 individual T cell–APC interactions were imaged for each T cell type over five separate experiments. C, 3D imaging of fixed cells showing specific accumulation of MHC:peptide and TCR at the interfaces of control and anergic T cells and APCs. D, En face view to display the spatial distribution of accumulated MHC:peptide and TCR, which colocalize at the center of the T cell–APC interface for both control and anergic T cells. E, Spatial distribution of ICAM-1 and GFP-tagged MHC:peptide at the T cell–APC interface. ICAM-1 forms a ring surrounding the central MHC:peptide. Images in C–E are representative of five separate experiments with >200 synapses imaged for both control and anergic T cells. Live-cell images (B) were collected with a 40× objective, and fixed images (C–E) were collected with a 60× objective. Scale bar, 10 μm.

The 3D interface (Fig. 3A). The total surface area of the T cell–APC interface region with GFP fluorescence intensity at least 2-fold higher than that of the noninterface regions of the APC membrane was summed. The data in Fig. 3B are representative of seven experiments performed with >500 control and anergic synapses imaged. For the control cells, the area of GFP ≥2-fold above background was 3.69 ± 0.35 μm² (mean ± SEM), whereas for the anergic cells it was 3.59 ± 0.38 μm². The total area of the accumulated MHC:peptide was slightly larger for the control synapses, but this slight difference was not statistically significant.

We next calculated the total integrated intensity of the GFP spots ≥2-fold above background across the control and anergic T cell–APC interfaces. The integrated intensity is a relative measure of the intensity of the GFP and is reflective of the total amount of material accumulated at the interface. The integrated intensity of GFP-tagged MHC:peptide at the control interfaces ≥2-fold above background was 4.757 ± 4.573, whereas for the anergic synapses it was 41.607 ± 39.05 (Fig. 3C). This indicated that, like the total area values, the amount of accumulated MHC:peptide at the T cell–APC interface was slightly higher for the control T cells, but the difference was not statistically significant.

During the analysis of the en face views of the 3D rotations for the area and integrated intensity calculations, we noted several different characteristic morphological patterns of the MHC:peptide distributions at the interface with the APC for both control and anergic T cells. We classified these into five categories of distribution: central spot, multifocal center, multifocal across the interface, edge of the interface, and no specific accumulation at the interface. Examples of the first four of these morphologies are shown in Fig. 3D. Calculated frequencies for each morphological pattern for one representative experiment of seven are shown in Fig. 3E. For both control and anergic cells, the majority of material was accumulated in a central location (61.6% for control cells and 60.7% for anergic cells), although there are slightly more anergic synapses with central spots and slightly more control synapses with multifocal center distributions. The frequency of the multifocal center distributions across the interface morphological pattern was also slightly higher for the control cells (30.8 versus 27.4% for anergic cells). The main difference between control and anergic cells was in the percentage of interfaces without specific MHC:peptide accumulation. Out of 127 control interfaces examined in this experiment, none lacked accumulation of the GFP-tagged MHC:peptide at the interface. In contrast, 7.6% of the 131 anergic interfaces imaged in this experiment lacked accumulation of the MHC:peptide at the interface. Taken together, the results in Fig. 3 show that there is specific accumulation of the GFP-tagged MHC:peptide complexes at the T cell–APC interface for both control and anergic T cells. The amount of material and the area of the interface that it occupies are slightly lower for the anergic...
cells, but the differences did not reach statistical significance. Additionally, the spatial distribution patterns of the accumulated MHC:peptide were similar for the control and anergic cells, although a small percentage of the anergic T cell–APC conjugates do not have accumulation of the MHC:peptide at the interface.

F-actin accumulation at the anergic synapse is slightly reduced, but spatial distribution is not altered

The results in Figs. 2 and 3 suggest that anergic T cells were making relatively normal immunological synapses. However, the MHC:peptide distribution is only one aspect of the immunological synapse. To further characterize the immunological synapses formed by anergic T cells, we stained control and anergic T cell–APC conjugates for several other molecules.

Actin polarization and spatial redistribution are critical for immunological synapse formation and formation of signaling microclusters (52–54). We began characterizing anergic synapse composition by looking at the total amount and spatial distribution of F-actin at the anergic synapse. Images of GFP-tagged MHC: peptide and pTyr at the T cell–APC interfaces of control and anergic T cells. Control cells have slightly less pTyr at the interface, but this difference is not significant. Accumulation of pERK 1/2 is significantly reduced at the anergic synapse. Images in are representative of four separate imaging experiments and >125 T cell–APC conjugates for each condition. There are two anergic cells associated with the APC in C whose immune synapses formed in separate optical sections. The accumulation of GFP is similar in both, but because they are in separate optical planes the GFP accumulation at both T cell–APC interfaces is not visible simultaneously in a single plane. Note that the intensity and brightness settings for each image in each panel are identical, so perceived differences in brightness are representative of differences in staining intensity.

FIGURE 3. The amount, spatial distribution, and total area of accumulated MHC:peptide at the anergic immunological synapse are similar to those of control synapses. A, DIC (left) and fluorescent images (center) of T cell–APC conjugates. The en face view of the T cell–APC interface is also shown (right). B and C. The total area (B) and integrated intensity (C) of accumulated MHC:peptide complexes ≥2-fold above background in en face views as in A. D. Examples of MHC:peptide morphology at the T cell–APC interface for both control and anergic T cells. E. Frequency of the distinct MHC:peptide accumulation morphologies identified in D. This is a single experiment representative of seven separate experiments with >500 total control and anergic synapses imaged. There were 127 control synapses and 131 anergic synapses in the experiment compared in E. All of the images were collected with a 60× objective. Scale bar, 10 μm.

FIGURE 4. At the anergic synapse, there is slightly increased accumulation of pTyr residues, slightly reduced F-actin accumulation, and significantly reduced pERK 1/2. A. Phalloidin staining showing slight reduction in the accumulation of F-actin to the anergic synapses. En face views (right) show that spatial distribution of accumulated F-actin is the same for control and anergic T cells. B. Images of GFP-tagged MHC:peptide and pTyr at the T cell–APC interfaces of control and anergic T cells. Control cells have slightly less pTyr at the interface, but this difference is not significant. C. Accumulation of pERK 1/2 is significantly reduced at the anergic synapse. Images in are representative of four separate imaging experiments and >125 T cell–APC conjugates for each condition. There are two anergic cells associated with the APC in C whose immune synapses formed in separate optical sections. The accumulation of GFP is similar in both, but because they are in separate optical planes the GFP accumulation at both T cell–APC interfaces is not visible simultaneously in a single plane. Note that the intensity and brightness settings for each image in each panel are identical, so perceived differences in brightness are representative of differences in staining intensity.
pTyr accumulation is slightly higher at the anergic immunological synapse, whereas pERK 1/2 levels are significantly lower.

We examined the spatial distribution of total pTyr as a measure of TCR signaling at the immunological synapse. As shown Fig. 4B, there was appreciable accumulation of pTyr to the interfaces of both control and anergic T cells. There was more pTyr at the anergic interface than at the control interface, although the difference in integrated intensity was not statistically significant ($p = 0.11$) (Fig. 4B). The en face views showed areas of increased MHC:peptide intensity adjacent to areas of increased pTyr intensity at the centers of the interfaces, and their positions correlated very well. This correlation was found in >90% of both control and anergic T cells.

We also examined the levels of pERK 1/2 accumulated at the T cell–APC interface in Fig. 4C. Similar to the results in Figs. 3, 4A, and 4B, the amount of accumulated GFP-tagged MHC:peptide at the anergic immune synapse formed by the T cell on top of the APC is very similar to the accumulation at the control synapse. Although there appear to be differences in the GFP accumulation between the two anergic T cells in Fig. 4C, their accumulation is nearly identical. The two T cells form mature immune synapses in different focal planes, and because the image in Fig. 4C is a single optical section, it is not possible to display both synapses simultaneously. The control T cell images in Fig. 4C clearly show accumulation of pERK at the T cell–APC interface. This accumulation was >2-fold above background staining in 93% of the control immune synapses. For the controls, there was a slight reduction in the pERK intensity in the area where the clustered cSMAC MHC:peptide was accumulated, consistent with recent models suggesting that the majority of signaling occurs in peripheral microclusters (6). By comparison, the accumulation of pERK at the anergic immunological synapse was significantly lower. The accumulated material only reached 1.25- to 1.5-fold above background in 79% of the anergic T cell–APC interfaces. Fewer than 5% of the anergic synapses had any areas that reached 2-fold above background, and where they occurred, these areas were very small. This reduction in pERK accumulation is supported by the reduced CD69 upregulation observed in Fig. 1C and is consistent with Adams et al. (55).

Trogocytosis by anergic T cells

A very common feature of these fixed T cell–APC conjugate images was the transfer of small amounts of GFP-tagged MHC:peptide from the APCs to the T cells. These small green spots were observed on the sides of the T cells in Fig. 4B (also visible in Fig. 2C). We saw this intercellular transfer, termed trogocytosis (56), on 85% of control conjugates and 82.4% of anergic conjugates. In the majority of these instances (52% for controls and 60.4% for anergic cells), the transferred material had moved to the distal pole of the T cell, in an area directly opposite the interface. The significance of this intracellular molecular transfer is unclear. Interestingly, there was accumulation of pTyr to the spots transferred onto the T cells, suggesting sustained signaling at the location of the transferred MHC:peptide. This is in agreement with our previous data showing trogocytosed MHC:peptide in association with pTyr on the surfaces of T cells that spontaneously dissociated from APCs (46).

At anergic immunological synapses, c-Cbl and Cbl-b are enhanced in the pSMAC and Cbl-b is also accumulated in the cSMAC

The increase in total pTyr and reductions in pERK 1/2 and F-actin accumulation suggest that there are differences in the signaling from MHC:peptide-engaged TCR in the anergic cells. This is consistent with previous reports showing signaling defects in anergic T cells (14). Potential negative regulators of TCR signaling that have been implicated in altering TCR signaling in anergic T cells include the E3 ubiquitin ligases Cbl-b, c-Cbl, and GRAIL (22). We examined the accumulation and spatial distribution of these molecules at the T cell–APC interfaces of control and anergic T cells. Our initial experiments found that GRAIL was not localized to the T cell–APC interface in either control or anergic T cells (data not shown), consistent with previous reports that have localized it to endocytic membranes (57). We did not characterize GRAIL distribution further.

There were significant differences between control and anergic T cells when the distributions of c-Cbl at the immunological synapses were compared. In the control T cells, the majority of cells (62.5%) had either no specific accumulation or exclusion from the T cell–APC interface region. This was slightly higher than what was found with the anergic T cells (54.5%). There was a small increase in c-Cbl in 18.8% of the control T cell–APC interfaces, but this increase did not reach a threshold of 2-fold above background. By comparison, the proportion of anergic T cells with c-Cbl accumulation at the T cell–APC interface was similar to that of the controls (18.2%), but the intensity of staining was significantly higher (Fig. 5A). In all of the anergic T cell–APC interfaces examined, there was accumulation ≥2-fold above background staining levels, whereas none of the control synapses reached that threshold.

There were very clear differences in the spatial distributions of c-Cbl at the control and anergic T cell–APC interfaces when the en face views were compared (Fig. 5B, 5C). c-Cbl is distributed across the interface in a multifocal pattern for 12.5% of the control synapses. This staining pattern was not observed with the anergic cells. For the majority of both control (64.8%) and anergic cells (83.4%), a ring of c-Cbl formed around the accumulated GFP-tagged MHC:peptide complexes. Interestingly, all of the anergic synapse c-Cbl rings reached ≥2-fold above background, but none of the control synapse c-Cbl rings reached that threshold. For the vast majority of both control and anergic synapses, there was either no accumulation of c-Cbl at the center of the ring or the c-Cbl was excluded from the ring (Fig. 5C). However, a fraction (28%) of the anergic synapses with a c-Cbl ring ≥2-fold above background also had specific accumulation of c-Cbl in the center of the ring that reached a level ≥1.5-fold above background colocalized with MHC:peptide. This distribution pattern was not seen with the control cells. Thus, c-Cbl appears to be preferentially accumulated in the pSMAC of both control and anergic synapses, but there was significantly more c-Cbl in the anergic immunological synapses.

The accumulation of Cbl-b was significantly different between anergic and control immunological synapses. Cbl-b levels were increased in virtually all of the control and anergic synapses (Fig. 5A), with the specific accumulation reaching a threshold of ≥2-fold above background in 94.4% of the anergic synapses, whereas only 8.3% of the control synapses reached this threshold (Fig. 5D).

The spatial distribution of Cbl-b at the control and anergic synapses was also significantly different (Fig. 5E). For the control cells, 25.1% of the immunological synapses had uniform, dim (<1.5-fold above background) Cbl-b staining across the interface, whereas in the anergic synapses this uniform staining was observed only 9.8% of the time (Fig. 5E). For the majority of control immunological synapses (74.9%), there was a dim ring of slightly enhanced Cbl-b staining (<2-fold above background) with either no enhanced central Cbl-b or exclusion of Cbl-b staining in the center of that ring (Fig. 5E). In 87% of these control synapses with a dim Cbl-b ring, the GFP-tagged MHC:peptide was found within the Cbl-b ring, and there was no colocalization of these two molecules (Fig. 5B). As with c-Cbl, this distribution pattern was...
suggestive of pSMAC localization for the Cbl-b in the control immunological synapses. In contrast to the control immunological synapses, 94% of the anergic immunological synapses had an intense ring of Cbl-b staining $2$-fold above background. Similar to the distribution of c-Cbl, this Cbl-b ring surrounds a central MHC:peptide region (Fig. 5B). In approximately two thirds of these anergic synapses with an intense Cbl-b ring (63.5%), there was also a dim central region of Cbl-b staining (1.25- to 1.5-fold above background) that colocalizes with the central MHC:peptide accumulation. For the remaining anergic interfaces (26.7%), there was an intense Cbl-b ring with enhanced Cbl-b staining ($\geq 2$-fold above background) in the center (Fig. 5E) that colocalizes with the accumulated central MHC:peptide (Fig. 5B).

The data in Fig. 5 clearly show that both c-Cbl and Cbl-b are accumulated to the T cell–APC interface in the majority of both control and anergic synapses. However, the amount accumulated at the anergic synapses is significantly higher. Both c-Cbl and Cbl-b are localized in ring-like structures surrounding centrally accumulated MHC:peptide complexes, which strongly suggests that these negative regulators of TCR signaling are found in the pSMAC. In the vast majority of anergic synapses, there was also some Cbl-b, but not c-Cbl, accumulation in the cSMAC that colocalized with the GFP-tagged MHC:peptide complexes and, by extension, the engaged TCR (Fig. 5B, Supplemental Movies 1, 2).

**Discussion**

Ag recognition by CD4$^+$ T cells in the absence of a costimulatory signal leads to the induction of anergy (13). Anergy is associated with alterations in intracellular signaling and resultant effector functions such as reductions of IL-2 and IFN-γ secretion (14–16, 58). The immunological synapse is important in sustained signaling (4) and delivery of a subset of effector cytokines (8) by CD4$^+$ T cells. In this report, we have examined the immunological synapse formed by anergic T cells to determine whether alterations in energized T cells are reflected in changes to the composition or structure of the immunological synapse.

The mature immunological synapse is characterized by a prototypical cSMAC with accumulated MHC:peptide, TCR, and PKCθ, among others, surrounded by a pSMAC enriched in molecules such as ICAM-1, LFA-1, and talin (1–3). We found that anergic cells form mature immunological synapses with specific accumulation of GFP-tagged MHC:peptide complexes at the center of the T cell–APC interface (Figs. 2E, 3D, 3E). The amount of accumulated MHC:peptide, the area of the T cell–APC interface covered by the accumulated MHC:peptide, and the spatial distribution of these molecules did not differ significantly between...
control and anergic synapses. In live-cell imaging experiments, we found that the kinetics of mature immunological synapse formation were similar for control and anergic T cells.

There is considerable debate in the literature as to the ability of anergic CD4+ T cells to form mature immunological synapses. Our results would appear to be inconsistent with the findings of Heissmeyer et al. (19) who showed in a planar lipid bilayer system that mature immunological synapses formed by ionomycin-energized CD4+ T cells were not stable and were disintegrating at 1 h (19). However, at shorter time points (≤22 min), anergic T cells formed mature synapses with an ICAM-1 ring surrounding accumulated central MHC:peptide (19), which is consistent with our findings. The possible instability of anergic synapses in our system may be reflected in lower frequencies of T cell–APC conjugates (Fig. 2A).

Ise et al. (39) also found significant reductions in TCR, PKC0, and cholera toxin B accumulation at the anergic T cell–APC interface. In contrast, Zambricki et al. (40) found that there were no significant differences in the accumulation of LFA-1 (a pSMAC marker) or PKC0 (a cSMAC marker) between anergic and control synapses after 18 h of T cell–APC coculture. Neither Zambricki et al. (40) or Ise et al. (39) examined the spatial distribution of molecules at the immunological synapse as we have done in this report. Our results would initially appear to be at odds with those of Carlin et al. (38) who showed with anergized human T cell lines that CD3ε and TCRζ were accumulated at the anergic T cell–APC interface predominantly in arc or ring-like structures after 30 min of T cell and APC coculture (38) rather than the predominantly central accumulation that we observed. This apparent discrepancy is likely due to differences in the level of specific MHC:peptide on the surface of the APC, because they state that at higher Ag doses anergic T cells showed a predominantly central accumulation of both CD3ε and TCRζ (38). Thus, our results are consistent with many of these earlier reports, even those that would initially seem to be at odds with our findings, and confirm that anergic CD4+ T cells do form mature immunological synapses.

After confirming that anergic T cells form mature immunological synapses, we examined these structures to further characterize the accumulation and spatial distribution of additional molecules. We found that slightly reduced levels of F-actin accumulated at the anergic immunological synapse, but the spatial distribution was normal. This is consistent with previous studies that showed sustained accumulation, and the correct location of the actin cytoskeleton is essential for formation and maintenance of the mature immunological synapse (52).

When we looked at molecules associated with intracellular signaling, we observed a slight, but not significant, increase in total pTyr accumulation at the anergic synapse, confirming that there was Ag recognition and TCR triggering in the anergic cells. In contrast, the level of pERK was significantly reduced at the anergic synapse. This potentially explains the reduced CD69 upregulation observed in anergic cells (Fig. 1), because CD69 expression is mediated by the ERK signaling pathway (59). Adams et al. (55) previously reported that pERK was found at the plasma membranes of activated control T cells after Ag recognition but remained cytoplasmic in anergic T cells. However, the low-resolution laser scanning cytometry technique that they employed was not able to determine potential changes at the T cell–APC interface (55). Our results show for the first time that there is a significant reduction in pERK at the anergic immunological synapse.

One of the most intriguing observations that we made was the enhanced accumulation of the E3 ubiquitin ligases c-Cbl and Cbl-b at the anergic synapses (Fig. 5). c-Cbl and Cbl-b are important negative regulators of TCR signaling (23, 28), and previous studies have shown that Cbl-b is a key regulator of anergy (20). These molecules have been observed to accumulate in the region of triggered TCRs on normal T cells (60, 61), but no previous studies have shown the accumulation and spatial distribution of Cbl-b and c-Cbl at the anergic immunological synapse. Our results show for the first time that both c-Cbl and Cbl-b are preferentially accumulated in the pSMAC of immunological synapses formed by T cells (Fig. 5), but the level of accumulation is significantly higher for anergic cells. In addition, for a majority of anergic T cells, Cbl-b is also enhanced in the cSMAC, whereas c-Cbl is found in the cSMAC in a minority of anergic immunological synapses. These molecules were not found at appreciable levels in cSMACs of control synapses. These findings have significant implications in the initiation or maintenance, or both, of the anergic state.

Triggering of the TCR by cognate MHC:peptide complexes leads to the activation of the Src family kinase Lck, which in turn phosphorylates and activates Cbl molecules (27, 62). This leads to the ubiquitination of components of the TCR complex (62). The TCR is downmodulated after triggering and trafficked to the lysosomes for degradation rather than being recycled to the plasma membrane (24, 63). This differential trafficking is a Cbl-dependent process that has been proposed to attenuate TCR-mediated signaling (24, 28, 63, 64). We observed increased TCR down-modulation of the TCR in anergic T cells (Fig. 1), which is consistent with previously observed increases in Cbl-b expression in anergic T cells (19, 21, 41). The levels of Cbl-b in the anergic cells used in this study are comparable to the levels of Cbl-b induced by the ionomycin-induced anergic state as described by Heissmeyer et al. (19) (data not shown).

Cbl molecules are key regulators of anergy (20), and our results are the first to demonstrate the preferential accumulation of these molecules at the anergic immunological synapse. Wiedemann et al. (61) have previously observed that Cbl-b, pTyr, and ubiquitin localized to Ab-triggered TCRs in human T cell clones, and Naramur et al. (24) proposed that Cbl-b and c-Cbl would destabilize the immunological synapse and terminate signaling. c-Cbl has been shown to control LAT microcluster persistence (64) and PLCγ1 degradation (65), and Heissmeyer et al. (19) showed that the degradation of PLCγ1 was mediated, at least in part, by Cbl-b, resulting in the disintegration of the pSMAC at anergic synapses (19). We have not examined the stability of control or anergic immunological synapses here. However, our Cbl-b and c-Cbl data support the previous reports focusing on the signaling and potential stability of the anergic immunological synapse. Combining our current observations with those previously published findings, we would like to propose a tentative model for the events occurring at the anergic immunological synapse. Ag recognition by the anergic T cells combined with the activation of integrin “inside-out” signaling triggered by Ag recognition (66) leads to formation of a tight anergic T cell–APC conjugate. Over the next several minutes, small microclusters of MHC:peptide-engaged TCRs form that coalesce into a mature immunological synapse with defined cSMAC and pSMAC domains. These microclusters in the nascent pSMAC have been shown to be the site of TCR signaling (6), and their persistence is controlled, in part, by c-Cbl–mediated ubiquitination (64). Cbl-b and c-Cbl are recruited to both control and anergic immunological synapses, but at the control synapse CD28 signaling leads to the ubiquitination and destruction of the Cbl molecules as shown by Li et al. (21). With the lack of a CD28 signal during anergy induction, Cbl molecules are not degraded, leading to significantly higher expression of Cbl-b and c-Cbl in the anergic cells. At the subsequent immunological synapse formed by these anergic cells, our data here show that Cbl-b and c-Cbl are preferentially accumulated compared with control T cells. At the anergic synapse, they may mediate ubiquitination of several molecules, including TCRζ,
PLCγ1, and PI3K p85. At time points later than the 30 min used in this report, the Cbl-mediated ubiquitination may result in the destruction of PLCγ1 and hypophosphorylation of Vav1, altering actin accumulation and distribution at the anergic synapse. The combination of actin disruption and cessation of PLCγ1-mediated signaling may result in immune synapse disintegration as the T cells reinitiate locomotion across the APC membrane as has previously been shown (19). Further experimentation will be necessary to confirm this model. Our findings that Cbl-b and c-Cbl preferentially accumulate at the anergic synapse and have altered spatial distribution provide critical information that aids in our understanding of the process of anergy maintenance in CD4+ T cells.

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Disclosures

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