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Cutting Edge: The Dendritic Cell Cytoskeleton Is Critical for the Formation of the Immunological Synapse[1]

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The binding of a T cell to an APC results in T cell actin cytoskeletal rearrangement leading to the formation of an immunological synapse. The APC cytoskeleton has been thought to play a passive role in this process. In this study, we demonstrate that dendritic cells (DC), unlike other APC, actively polarize their actin cytoskeleton during interaction with T cells. DC cytoskeletal rearrangement was critical for both the clustering and the activation of resting T cells. This study provides compelling evidence that the APC cytoskeleton plays an active role in the immunological synapse and may explain the unique ability of DC to activate resting T cells. ***The Journal of Immunology,*** **2001, 166: 1452–1456.**

Sustained TCR engagement, which is important for productive T cell activation, depends on the formation of an immunological synapse (1–3). This specialized contact between the T cell and the APC is dependent on reorganization of the T cell actin cytoskeleton and is characterized by the accumulation of filamentous actin (F-actin)[3] and other cytoskeletal proteins in the T cell at the contact point with the APC (4, 5). These active changes in the T cell cytoskeleton result in the dynamic clustering of T cell surface receptors and signaling molecules at the interface with the APC (3, 6). Inhibition of T cell cytoskeletal rearrangement with cytochalasin D (CytD), which prevents F-actin formation (7), prevents surface receptor clustering, T cell proliferation, and IL-2 production (8–10). Overall, the changes in the cytoskeleton likely serve to increase the contact between TCR and MHC and provide the optimal environment for signaling molecules downstream of the TCR (11).

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[3] Abbreviations used in this paper: F-actin, filamentous actin; DC, dendritic cell; CytD, cytochalasin D.

Although active T cell cytoskeletal rearrangement is critical for the formation of the immunological synapse, the APC cytoskeleton has been thought to play a passive role (1). When B cells are used as APC, no polarization of their cytoskeleton has been observed during interaction with T cells (4) and inhibition of APC cytoskeletal rearrangement has no effect on APC-T cell binding or T cell activation (8). Indeed, the rearrangement of surface receptors on the APC has been thought to be a passive event and can be demonstrated using lipid bilayers as surrogate APC (3).

Although other APC can stimulate activated T cells, the specialized ability of dendritic cells (DC) to cluster and activate resting T cells suggests that the immunological synapse between DC and resting T cells is substantially different (12). In this study, we demonstrate that in contrast to the results seen with B cells, DC actively polarize F-actin and fascin, an actin-bundling protein, during clustering with T cells. This DC actin cytoskeletal rearrangement was critical for the clustering and activation of resting T cells, indicating an important role for the DC cytoskeleton in the establishment of the immunological synapse.

Materials and Methods

Abs and reagents

The anti-fascin mAb (mouse IgG1) was purchased from Dako (Carpinteria, CA). Anti-CD4 (GK1.5; rat IgG2b), anti-Thy1.2 (CD90; mouse IgG2b), anti-CD11c (N418; hamster IgG), and FITC/PE-conjugated secondary Abs were purchased from Cedarlane Laboratories (Hornby, Ontario, Canada). Alexa 488 (green) and 594 (red) goat anti-mouse IgG and Alexa Fluor 568 (red) phalloidin were purchased from Molecular Probes (Eugene, OR). CytD (Sigma-Aldrich, St. Louis, MO), jasplakinolide (Molecular Probes), and latrunculin A (Biomol, Plymouth Meeting, PA) were dissolved in DMSO.

Cell culture and purification

DC were prepared from BALB/c bone marrow as previously described using recombinant murine GM-CSF and LPS (13). The resulting DC were >85% CD11c, MHC class II, and B7-2 positive by flow cytometry.

Resting $CD4^+$ T cells from C57BL/6 or BALB/c spleen were negatively selected using a CD4 cell column (Cedarlane Laboratories). CD4 purity was >90% by flow cytometry. Activated $CD4^+$ T cells were generated by treating $CD4^+$ T cells with PMA (15 ng/ml) and ionomycin (500 ng/ml) for 48 h.

DC-T cell cluster analysis

DC or T cells were treated with 20 μM CytD or latrunculin A or 10 μM of jasplakinolide for 1 h at 37°C and washed three times before mixing. DC were centrifuged with syngeneic or allogeneic (resting or activated) T cells (1:3) at 50 × *g* for 5 min at 4°C in a conical tube. After centrifugation, the cells were incubated at 37°C in a water bath for 30 min and then resuspended and plated on poly-L-lysine (Sigma, St. Louis, MO)-coated slides.

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DC were also cocultured with allogeneic T cells at a ratio of 1:3 in flat-bottom 96-well plates and clusters were harvested at 0.5, 1, 2, 6, and 24 h. Slides were fixed in 10% acetate-buffered Formalin and permeabilized in 0.1% Triton X-100 for 10 min at room temperature before incubation with anti-fascin Ab. Alexa 488 (green) goat anti-mouse IgG was then used to detect fascin. For double staining with F-actin, slides were further incubated with Fluor 568 (red) phalloidin for 30 min. Fluorescence signals were detected with a Zeiss LSM510 confocal laser scanning microscope. Conjugates that had T cells binding to only one-half of the DC were captured. The DC cytoskeleton was scored as polarized if the intensity of F-actin was greater in the region adjacent to the binding T cell than in unbound regions. At least 50 conjugates were evaluated blindly in each treatment group.

DC were labeled with CellTracker green CFMDA (Molecular Probes) before mixing with prelabeled (CellTracker CM-Dil dye) $CD4^+$ T cells. Cocultures were examined using an inverted fluorescent microscope and DC-T cell clusters (defined as DC binding to one or more T cells) were expressed as the percentage of binding DC as follows: percent binding DC = (clustered DC/total DC) × 100.

Mixed lymphocyte reaction

DC or T cells or both were treated with graded doses (2–40 μM) of CytD or latrunculin A for 1 h at 37°C and washed three times. DC were treated with 25 μg/ml mitomycin C (Sigma-Aldrich) and added to 2×10^5 allogeneic $CD4^+$ T cells for 4 days in U-bottom 96-well plates. T cell proliferation was assessed by thymidine incorporation during the last 18 h.

Statistics

Statistical significance was assessed using a one-way ANOVA (GraphPad InStat; GraphPad, San Diego, CA). Where not significant indicates a p value of >0.05, $*$ indicates a p value <0.05, $**$ indicates a p value <0.01, and $***$ indicates a p value <0.001.

Results

Focal DC cytoskeletal polarization during interaction with allogeneic T cells

Polarization of surface receptors would not be indicative of an active process because it occurs in B cells and surrogate APC (3). Therefore, we evaluated the role of the DC cytoskeleton during interaction with allogeneic $CD4^+$ T cells by examining the localization of F-actin and fascin. Fascin is an actin-bundling protein that is expressed primarily in mature DC and has an important role in dendrite formation (14) and T cell activation (15). F-actin (Fig. 1, *a* and *b*) and fascin (Fig. 1, *c* and *d*) were uniformly distributed around the periphery of the cell in unclustered DC. When DC were clustered with $CD4^+$ T cells, there was a pronounced focal polarization of both F-actin (Fig. 1, *e* and *f*) and fascin (Fig. 1, *g* and *h*) toward the contact site with the T cells. Colocalization of fascin, which is only expressed in DC, with F-actin demonstrated that the F-actin was accumulating in the DC not just the T cell at the point of contact (Fig. 1*i*). All clustered DC in the cocultures were scored as polarized or nonpolarized by comparison of F-actin distribution in T cell contact areas with areas that did not contact T cells. Focal polarization occurred as early as 30 min and was present at all time points examined in the cocultures (0.5, 1, 2, 6, and 24 h). These results demonstrate that unlike other APC, DC are actively involved in formation of the immunological synapse through rearrangement of their actin cytoskeleton.

DC are potent at clustering syngeneic T cells in an Ag-independent manner which likely allows sampling of MHC peptide by the T cell (16). When syngeneic T cells clustered with DC, there was very little polarization of F-actin (Fig. 2*a*) or fascin (Fig. 2*b*). Focal polarization of DC F-actin was present in 84% of the clustered allogeneic DC group but only in 22% of syngeneic DC (Fig. 2*c*). Thus, the formation of the immunological synapse is an Ag-dependent event for both T cell and DC.

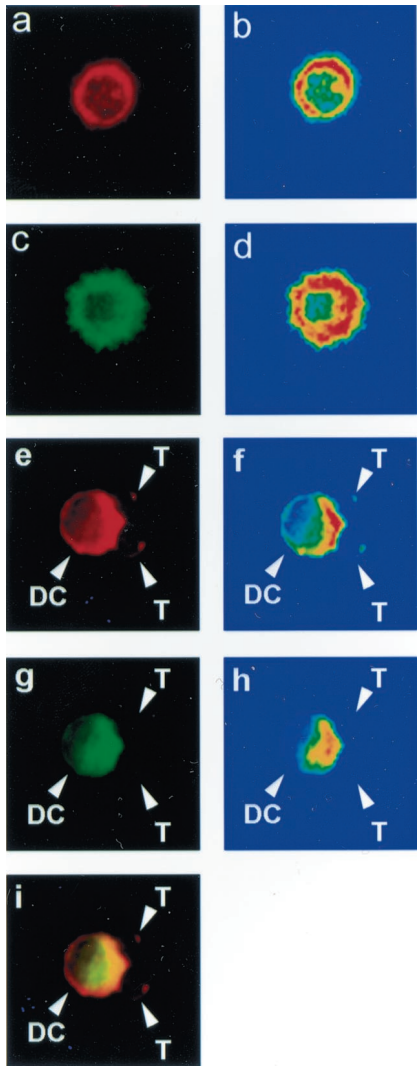

FIGURE 1. *a–i*, DC clustering allogeneic $CD4^+$ T cells show polarization of fascin and F-actin. DC were clustered with allogeneic $CD4^+$ T cells by centrifugation at low speed in a conical tube followed by incubation at 37°C for 30 min. Unclustered DC or DC clustering $CD4^+$ T cells were allowed to settle on poly-L-lysine-coated slides. Slides were then fixed, stained, and examined under confocal microscopy for the distribution of F-actin and fascin. F-actin (*a*) and fascin (*c*) localization in unclustered DC. F-actin (*e*) and fascin (*g*) polarization in DC clustering $CD4^+$ T cells. Copolarization of fascin and F-actin (*i*) in DC clustering $CD4^+$ T cells. *Right column* micrographs (*b*, *d*, *f*, and *h*) indicate the staining intensity (blue to red = low to high).

The DC actin cytoskeleton is important for the clustering of resting T cells

To evaluate the functional significance of DC actin cytoskeletal rearrangements, we examined the effects of inhibiting DC F-actin formation with CytD before the clustering of DC and T cells. CytD is a cell-permeable fungal toxin which is a potent inhibitor of F-actin formation and cytoskeletal function (7), and inhibition of T cell actin polymerization with CytD prevents immunological synapse formation (8). We generated clusters by centrifuging DC and T cells together at low speed and clusters were counted at 30 min. The level of F-actin in the CytD-pretreated DC was reduced by 47%, as assessed by Western blot at 1 h after the treatment, compared with control DC (data not shown). When T cells were pretreated with CytD, their ability to cluster with DC was reduced by 46% as has been previously described (Fig. 3*a*) (8). Importantly,

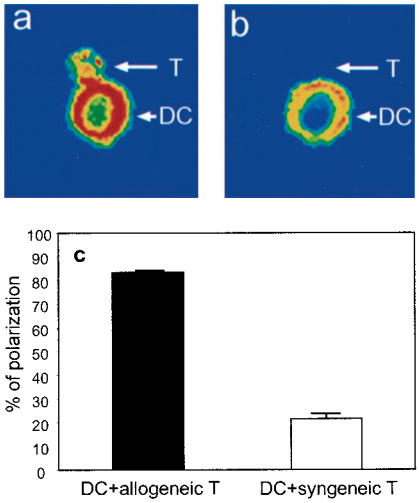

FIGURE 2. *a–c*, DC clustering syngeneic CD4$^+$ T cells have markedly reduced F-actin and fascin polarization. DC were clustered with syngeneic CD4$^+$ T cells by centrifugation at low speed in a conical tube followed by incubation at 37°C for 30 min. Clusters were then evaluated for F-actin and fascin localization under confocal microscopy. Distribution of F-actin (*a*) or fascin (*b*) was observed in DC clustering syngeneic CD4$^+$ T cells. The percentage of DC with F-actin polarization toward the T cell (*c*) during interaction with either allogeneic or syngeneic CD4$^+$ T cells. At least 50 conjugates were evaluated in each treatment group in three independent experiments.

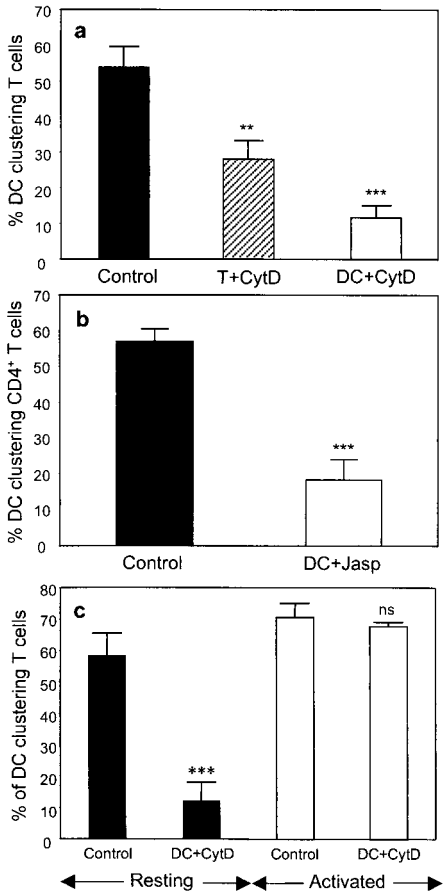

FIGURE 3. *a–c*, Pretreatment of DC with CytD inhibits their ability to cluster resting but not activated allogeneic CD4$^+$ T cells. DC were labeled with green CFMDA dye and CD4$^+$ T cells with red CM-Dil dye. *a*, Control or CytD (20 μM)-pretreated DC were mixed with control or CytD (20 μM)-pretreated resting CD4$^+$ T cells. *b*, Control or jasplakinolide (Jasp; 10 μM)-pretreated DC were mixed with resting CD4$^+$ T cells. *c*, Control or CytD (20 μM)-pretreated DC were mixed with either resting or activated CD4$^+$ T cells. Clusters were formed by low-speed centrifugation of DC and T cells as described in *Materials and Methods* and then transferred to a 96-well flat-bottom plate for counting. The clusters were counted immediately using an inverted fluorescence microscope. The results are expressed as the mean ± SD of the percentage of the DC clustering CD4$^+$ T cells and are representative of three independent experiments.

pretreatment of DC with CytD reduced clustering with untreated T cells by 76% (Fig. 3*a*). This is in direct contrast to similar experiments conducted previously using B cells as APC in which pretreatment of the B cells with CytD had no effect on cluster formation or T cell activation (8). In our hands, the overall clustering of B cells with resting allogeneic CD4$^+$T cells was very poor, with no significant difference seen in the percentage of B cells clustered between the control (3.5 ± 2.5%) and the CytD-pretreated group (4.1 ± 1.4%). To confirm that the observed inhibition was due to the effect of CytD on DC actin cytoskeletal rearrangement, DC were also pretreated with jasplakinolide, which prevents actin rearrangement by stabilizing the F-actin rather than inhibiting its formation (17). Pretreatment of DC with jasplakinolide reduced clustering with untreated T cells by 68% (Fig. 3*b*). Clustering of syngeneic T cells was not affected by pretreatment of DC with CytD (data not shown), which is consistent with the lack of polarization observed in the DC in syngeneic DC-T cell clusters.

We next evaluated the functional effects of the DC actin cytoskeleton on the clustering of resting or activated T cells. DC are much more potent at clustering resting T cells than other APC; however, activated T cells are capable of clustering with B cells and macrophages as well as with DC (12). Clustering of resting T cells was significantly inhibited by pretreatment of DC with CytD, whereas clustering of activated T cells was not significantly inhibited (Fig. 3*c*). Similar results were seen with latrunculin A (18), which inhibits actin cytoskeletal rearrangement by a different mechanism (data not shown). This suggests that although activated T cells can form an immunological synapse with any APC, resting T cells require active participation of the DC cytoskeleton.

Inhibition of the DC actin cytoskeleton prevents T cell activation

To evaluate the role of the DC actin cytoskeleton in T cell activation, we performed a MLR after pretreatment of DC or T cells or both with CytD. Consistent with previous studies, pretreatment of the T cells significantly inhibited the MLR (8). However, we found that inhibition of MLR was greater when the DC were pretreated (Fig. 4*a*). DC treated with CytD or latrunculin A for 1 h did not differ significantly compared with untreated DC in the expression of MHC class II, B7-2, or fascin as detected by flow cytometry (data not shown). MLR inhibition was seen at all doses of CytD used (Fig. 4*b*) and at different stimulator to responder ratios (Fig. 4*c*). There was also a dose response with greater doses of CytD resulting in greater inhibition of T cell proliferation (Fig. 4*b*). There was a strong correlation ($r = 0.99$) between the clustering seen and the degree of T cell activation seen in the different treatment groups. Pretreatment of DC with latrunculin A resulted in a similar inhibition of T cell activation (data not shown). In contrast to DC, the overall proliferation of resting CD4$^+$ T cells with B cells was very poor, consistent with the clustering data, but there was no significant difference between the control and the CytD-pretreated B cell group (data not shown).

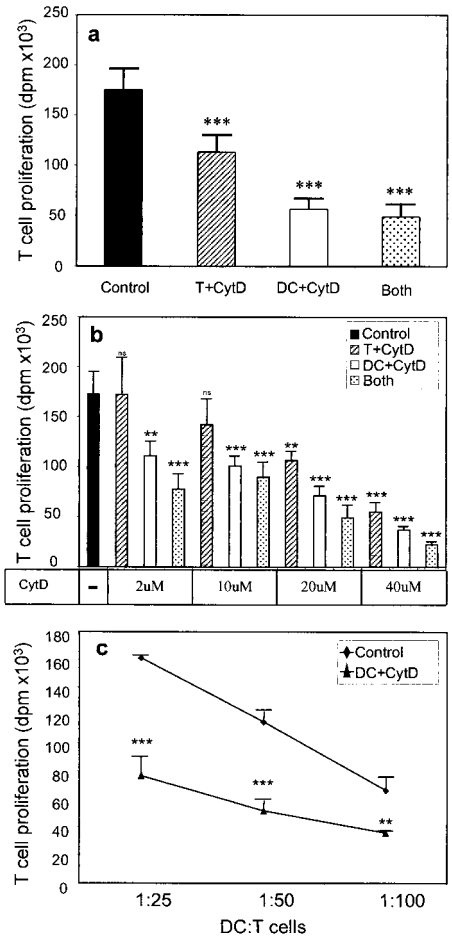

FIGURE 4. *a–c*, Pretreatment of DC with CytD inhibits their ability to activate $CD4^+$ T cells. *a*, Control or CytD (20 μM)- pretreated DC were mixed with control or CytD (20 μM)-pretreated $CD4^+$ T cells. *b*, Either DC or $CD4^+$ T cells or both were pretreated with different doses of CytD (2–40 μM) before mixing. *c*, Control or CytD (20 μM)-pretreated DC were mixed with $CD4^+$ T cells at different DC:T cell ratios. The cells were pulsed with [^{3}H]thymidine in the last 18 h of the 4-day MLR. T cell proliferation was assessed by measuring the [^{3}H]thymidine uptake in a liquid scintillation counter. The results are expressed as mean dpm ± SD and are representative of five independent experiments.

Discussion

In this report, we demonstrate that the DC actin cytoskeleton focally polarizes during the clustering of DC with $CD4^+$ T cells. This focal polarization involves the accumulation of F-actin and the actin-bundling protein, fascin, at the interface between the DC and the T cell. Inhibitors of DC actin polymerization reduce the clustering of resting T cells as well as their activation. These data indicate that, unlike with other APC, the DC actin cytoskeleton plays an active role in the establishment of the immunological synapse.

The involvement of the T cell actin cytoskeleton in maintaining an immunological synapse with the APC has been well established (4). The APC used in these studies have been mostly B lymphocytes which are much less potent than DC at clustering and activating T cells (12). No cytoskeletal reorganization occurs in the B cells used in these studies and inhibition of the B cell cytoskeleton with CytD does not affect their ability to cluster and stimulate T cells (1, 8). We have confirmed these findings in our system.

DC have several unique features which suggest that they play a more active role during their interactions with resting T cells (19). Early studies demonstrated that DC were able to cluster resting T cells in both an Ag-dependent and -independent fashion, whereas other APC could not (16). DC-SIGN has recently been identified as a DC-restricted receptor responsible for the Ag-independent clustering of resting T cells (20). We now demonstrate that DC actin cytoskeletal polarization is important for the Ag-dependent cluster formation and activation of resting T cells. In contrast, although the DC cytoskeleton polarizes during interaction with activated T cells (data not shown), this polarization is not necessary for clustering or activation. This is consistent with previous studies which demonstrated that B cells, which do not polarize their cytoskeleton, could cluster activated T cells as well as DC (1, 12). Thus, actin cytoskeletal rearrangement by DC leading to formation of the immunological synapse must be added to the list of specialized DC functions that allow for activation of resting T cells.

It remains to be demonstrated how the DC actin cytoskeleton participates in the clustering of resting T cells. Rearrangement of the DC cytoskeleton may align the DC with the T cell increasing contact area and adhesive interactions. Scanning electron micrographs of DC-T cell clusters have shown the DC dendrites wrapped around the T cell (21). The recruitment of fascin to the interface may suggest a role for dendrite formation in the contact area, since we and others have found that fascin is integral to the formation of dendrites (14) and activation of T cells (15). In addition, active rearrangement of the actin cytoskeleton may be important for activation of certain integrins, including LFA-1 (22), which are present on the DC (20) and participate in the clustering of resting T cells (20, 23). The signals involved in DC actin cytoskeleton rearrangement are under active investigation in our laboratory and will provide further insights into the function of these unique cells in the primary immune response.

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