Inefficient Cell Spreading and Cytoskeletal Polarization by CD4<sup>+</sup>CD8<sup>+</sup> Thymocytes: Regulation by the Thymic Environment

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Inefficient Cell Spreading and Cytoskeletal Polarization by CD4\textsuperscript{+}CD8\textsuperscript{+} Thymocytes: Regulation by the Thymic Environment\textsuperscript{1}

Eric Hailman and Paul M. Allen\textsuperscript{2}

CD4\textsuperscript{+}CD8\textsuperscript{+} double-positive (DP) thymocytes express a lower level of surface TCR than do mature T cells or single-positive (SP) thymocytes. Regulation of the TCR on DP thymocytes appears to result from intrathymic signaling, as in vitro culture of these cells results in spontaneous TCR up-regulation. In this study, we examined cell spreading and cytoskeletal polarization responses that have been shown to occur in response to TCR engagement in mature T cells. Using DP thymocytes stimulated on lipid bilayers or nontransgenic thymocytes added to anti-CD3-coated surfaces, we found that cell spreading and polarization of the microtubule organizing center and the actin cytoskeleton were inefficient in freshly isolated DP thymocytes, but were dramatically enhanced after overnight culture. SP (CD4\textsuperscript{+}) thymocytes showed efficient responses to TCR engagement, suggesting that releasing DP thymocytes from the thymic environment mimics some aspects of positive selection. The poor translation of a TCR signal to cytoskeletal responses could limit the ability of DP thymocytes to form stable contacts with APCs and may thereby regulate thymocyte selection during T cell development. The Journal of Immunology, 2005, 175: 4847–4857.

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\textsuperscript{3} Abbreviations used in this paper: DP, double positive; IRM, interference reflection microscopy; MTOC, microtubule organizing center; SP, single positive.
In this study, we examined the potential of DP thymocytes to translate ligation of the TCR to cytoskeletal responses. We were particularly interested in whether constitutive signaling in the thymus, which causes $\xi$ phosphorylation and contributes to low TCR expression, would limit the ability of DP thymocytes to spread and polarize in response to a TCR signal. To address this question, we took advantage of the findings of Nakayama et al. (5) that reported in vitro culture of DP thymocytes led to spontaneous TCR up-regulation and decreased $\xi$ phosphorylation, which would be changes expected to facilitate responses to TCR engagement. Although short-term culture was shown to lead to enhanced calcium influx in DP thymocytes (5, 23), no other functional responses have been shown to be altered after this treatment.

We measured MTOC polarization and cell spreading using two experimental systems in which the nature of the stimulus through the TCR or CD3 was well defined: TCR transgenic DP thymocytes interacting with lipid bilayers and nontransgenic thymocytes plated on Abs to CD3. In both systems, we found that cell spreading and MTOC polarization were relatively inefficient in freshly isolated DP thymocytes, but became dramatically more efficient after overnight culture. We also found that polarization of F-actin toward immobilized anti-CD3 in DP thymocytes became more efficient after overnight culture. Fresh SP thymocytes showed relatively efficient strong spreading and MTOC polarization in response to CD3 ligation. These responses were not enhanced after culture, suggesting that poor cytoskeletal responses are unique to the DP stage of development and that release from the thymic environment mimics some aspects of positive selection. Our results suggest that TCR regulation by constitutive signaling in the thymus limits the ability of DP thymocytes to polarize and form tight contacts in response to a TCR signal. The regulation of cytoskeletal responses of DP thymocytes may affect trafficking in the thymus by inhibiting stable contacts with APCs, and the inhibition of TCR signaling may have a more general effect of fine-tuning thymic selection.

Materials and Methods

Mice and cells

Thymocytes were from C57BL/6 mice or from TCR transgenic mice expressing the 3A9 TCR on an H-2 b RAG-1-deficient background (25). The 3A9 TCR recognizes I-Ak with hen egg lysozyme (HEL) 8–6 2peptide (24).

RPMI 1640 with 10% FCS. Cells used as “freshly isolated” were kept at frosted glass slides, and passed through a cell strainer (BD Falcon) to make CD8 (BD Pharmingen), and biotinylated anti-bovine BD Pharmingen, and anti-CD11a (M17/4; BioLegend). Abs used in imcyte populations, for 30 min at room temperature in PBS.

1/500 dilution, together with anti-CD4 and anti-CD8 to distinguish thymocytes populations, for 30 min at room temperature in PBS.

Cell assays and microscopy

Lipid bilayers containing GPI-linked I-A^k-HEL and ICAM-1 were prepared as described (25, 26). Briefly, bilayer proteins were purified from transfected cell lines by affinity chromatography with mAbs and incorporated into phosphatidylcholine vesicles, and lipid bilayers were formed by incorporating mixtures of these vesicles on glass coverslips (precleansed with chromic sulfuric acid) in parallel plate flow chambers (Biotech) for 10 min, followed by washing and blocking in HEPES-buffered saline with 1% human serum albumin (HBS/HSA). Cells (12–15 $\times$ 10^6 per flow chamber in HBS/HSA) were incubated on lipid bilayers at 37°C for 30 min then fixed and stained as previously described.

For assays on mAb-coated surfaces, mAbs (100 $\mu$g/ml in PBS, 2-µl spots) were incubated on precleansed glass coverslips for 2 h at room temperature in flow chambers, then washed and blocked with HBS/HSA. Cells (12–15 $\times$ 10^6 per flow chamber in HBS/HSA) were incubated on mAbs for 15 min then fixed and stained as described.

Fluorescence microscopy for most experiments used a Zeiss Axiovert microscope with a 100× Plan-Neofluor objective (Zeiss) mounted on a piezoelectric device (LVZRTZ position servo-controller; Physik Instrumente), xenon arc lamp, motorized stage (Ludl Electronic Products), motorized filter wheels for illumination and emission (Ludl Electronic Products), and appropriate dichroic and filter sets. Images were obtained with a Photometrics CoolSnap ES cooled CCD camera (Roper Scientific) controlled by IPLab imaging software (Scanalytics). For fluorescence images, exposures of 1–2 s with 2×2 binning were used.

Images of F-actin staining were obtained using a Zeiss LSM 510 confocal microscope with a 63×/1.4 oil objective. Sections $\leq$1.2-µm thick (pinhole 166 µm) were obtained at the focal plane of the cell contact with the substrate, determined by focusing using interference reference microscopy (IRM), and then at 1-µm intervals through the cell.

Image processing and quantitation

Images were processed using IPLab imaging software. For most experiments, flatfield images were derived by averaging ≥20 images and used to correct for uneven illumination of experimental images with corrected as: experimental image/flatfield image $\times$ average intensity of flatfield. Contact formation by TCR transgenic DP thymocytes on bilayers was determined by examination of flatfield-corrected IRM images. Cell contacts were defined as dark regions $\approx$2 µm in smallest diameter. To exclude double-negative thymocytes or other cell types, only contacts correlating with cells staining positively for CD8 were included in the analysis. For thymocytes forming contacts on mAb-coated coverslips, the contact areas were difficult to define using IRM images, particularly for DP thymocytes on anti-CD3 (see Results). Instead, we defined the contact area by manually tracing the outline of each cell from overlays of images of cells stained for CD4 and CD8. Contact areas of individual cells were then determined from cell outlines using the segmentation measurement functions of IPLab. For histogram data, contact areas of individual cells were rounded up to the next 5 µm^2; thus, a data point at 15 µm^2 represents all cell contacts $>10$ µm^2 and $\leq$15 µm^2. Cells were defined as DP or CD4 + or CD8 + SP thymocytes by examining images of CD4 and CD8 fluorescence individually and scoring cells as positive or negative for each marker.

MTOC polarization was assessed from fluorescence images of cells stained for $\alpha$-tubulin taken at the focal plane of the contact with the substrate. Only cells that had a defined contact area, as already described, were included in this analysis. Cells were scored as polarized if the MTOC could be identified within this focal plane, with microtubules visibly radiating from a focal point separated from the edge of the cell.

For F-actin staining, images obtained using the confocal microscope were processed using IPLab imaging software, without flatfield correction. Z-stacks of images of actin staining were converted to X-Z projections using the 3D Project extension of IPLab software. Projections were pseudocolored to reveal relative intensity of staining within each cell, using IPLab blue-green-red scale. Polarization toward the Ab-coated substrate was assessed from pseudocolored images. Three independent observers were asked to rate each cell as unpolarized, partially polarized, or polarized, based on the relative intensity of staining within 2 µm of the substrate (the bottom two rows in the pseudocolored X-Z projections) compared with the rest of the cell. Images of freshly isolated and overnight cultured DP thymocytes were presented to the observers intermixed and in random order, without identification of the category (fresh vs cultured) for each cell. The final rating for each cell was based on majority or average rating by the three observers. DP thymocytes from three randomly selected microscopic fields, totaling ≥50 cells for each condition, were used in the analysis.

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MTOC polarization in DP thymocytes stimulated on lipid bilayers

To assess the ability of DP thymocytes to polarize their MTOCs in response to antigenic stimulation, we added TCR transgenic thymocytes to glass-supported lipid bilayers containing peptide-MHC complex and ICAM-1, expressed as GPI-linked molecules (26). We used cells from mice transgenic for the 3A9 TCR, which recognizes the peptide-MHC complex of HEL48–62/I-Ak. 3A9 was expressed in RAG-1-deficient mice of the H-2b haplotype, resulting in a halt of T cell development at the DP stage due to a lack of positive selection; hence, thymi from these mice contain >85% DP and no SP thymocytes (25).

As we have shown previously (25), freshly isolated 3A9 DP thymocytes incubated on bilayers containing cognate peptide-MHC complexes and ICAM-1 adhered and spread on the bilayer, forming a close contact with the lipid coverslip, as seen on a dark region on IRM images (Fig. 1A, left). To determine the position of the MTOC, we fixed the cells after 30 min of incubation and detected microtubules by indirect immunofluorescence (Fig. 1A, right). In some cells, microtubules could be seen radiating from a central point in the plane of the contact of the cell with the lipid bilayer, indicating that the MTOC was polarized toward the TCR stimulus. In many cells, however, microtubules could be seen traversing the contact area, but the MTOC was not visible at the plane of the lipid bilayer (e.g., five of the six cells in Fig. 1A), suggesting that the cells were not polarized toward the bilayer. To confirm that the MTOC could be visualized in all cells, we obtained images of microtubules at increasing distances from the bilayer (Fig. 1B). In cells that had a polarized MTOC, microtubules radiated outward from the center of the contact zone with the bilayer, then followed the plasma membrane upward toward the distal pole of the cell (Fig. 1B, top). In cells where the MTOC was not visible at the plane of the bilayer, it was consistently found at a more distant site, either at the opposite pole from the synapse or along the side of the cell (Fig. 1B, bottom). Based on these results, we concluded that examining a single fluorescent image taken at the level of the bilayer was sufficient to assess MTOC polarization.

MTOC polarization in DP thymocytes is enhanced by in vitro culture

Images such as the one shown in Fig. 1A suggested that MTOC polarization by freshly isolated DP thymocytes was relatively inefficient: many cells received a signal through the TCR sufficient to cause formation of a close contact with the bilayer (an Ag-dependent process, see below) but insufficient to induce MTOC polarization. To determine whether the low TCR expression and constitutive ζ phosphorylation characteristic of DP thymocytes prevented efficient MTOC polarization, we cultured the cells in vitro before adding them to bilayers. This treatment has been shown to diminish ζ phosphorylation, increase TCR expression, and lead to more efficient TCR signaling, as judged by increased calcium flux upon receptor cross-linking (5). We subjected DP thymocytes to overnight culture, which led to an increase in TCR expression, as expected (Fig. 1C). This treatment did not appear to complex (pMHC) and a constant density of ICAM-1, and MTOC polarization was quantitated by scoring each cell for the presence of the MTOC within the contact area with the bilayer. At lower Ag densities, too few cells formed contacts to allow quantitation of MTOC polarization. Data represent mean ± SEM from two or three experiments for each data point; at least 100 cells for each condition were examined in each experiment. Error bars are omitted when the error was smaller than the symbol.
activate the cells, as there was no increase in CD69 expression (Fig. 1C) or cell size (by forward scatter, data not shown). However, cells added to bilayers after culture showed a dramatic increase in the efficiency of MTOC polarization, with a large majority of cells having well-organized, symmetrical MTOCs clearly visible near the center of the synapse, with individual microtubules visibly radiating to the edges of the contact area (Fig. 1D).

To quantitate the increase in MTOC polarization after overnight culture, we added fresh or overnight-cultured DP thymocytes to lipid bilayers containing increasing densities of peptide-MHC complex and a constant density of ICAM-1, and fixed and stained the cells as described. We assessed MTOC polarization in each cell that formed an IRM contact above a minimum size (see Materials and Methods) and that stained positively with a mAb to CD8 (to distinguish DP from double-negative thymocytes). Cells were considered polarized if the MTOC was visible at the focal plane of the bilayer. MTOC polarization by freshly isolated DP thymocytes was induced by Ag in a dose-dependent manner, but it was inefficient, with a high density of Ag (200 peptide-MHC/μm²) necessary to consistently induce polarization in the majority of cells (Fig. 1E). At lower Ag concentrations (2 or 20 peptide-MHC/μm²), cells adhered but the percentage that had polarized MTOCs was relatively low (20–60%). In previous work, we have used the same range of Ag densities to study receptor patterning in freshly isolated DP thymocytes, and found that a multifocal immunological synapse was formed under these conditions (25).

When DP thymocytes were cultured overnight before being added to bilayers, the efficiency of MTOC polarization was dramatically increased over a wide range of Ag densities. Polarization in cultured thymocytes was consistent and robust, with >80% of cells consistently displaying well-organized MTOCs at the synapse over a 1000-fold range of Ag concentrations in three independent experiments (Fig. 1E). We considered the possibility that cold-induced depolymerization of microtubules might inhibit MTOC polarization in freshly isolated thymocytes, which were incubated on ice before testing in vitro. Therefore, in some experiments, overnight-cultured thymocytes were stored on ice for at least 3 h before adding them to bilayers; this manipulation did not inhibit the efficient MTOC polarization shown in Fig. 1E. In addition, microtubules and MTOCs were visible in all freshly isolated DP thymocytes, whether or not they were polarized (Fig. 1B), indicating that the inefficient MTOC polarization in freshly isolated DP thymocytes was not due to cold-induced microtubule depolymerization.

These findings show that the lipid bilayer system provides sufficient antigenic stimulation to induce cytoskeletal polarization, and that the limited polarization in fresh DP thymocytes is due to an intrinsic property of the cells, rather than a limited strength of stimulation in the bilayer. The dramatic effect of short-term culture on polarization suggests that constitutive signals received by DP thymocytes in the thymus limit their ability to polarize in response to a TCR signal, and that in vitro culture releases the cells from this inhibition, allowing efficient translation of TCR ligation to a cytoskeletal response.

Contact formation by DP thymocytes is enhanced by in vitro culture

We asked whether the effect on the ability of cultured DP thymocytes to polarize was specific to a pathway leading to microtubule organization, or whether other TCR-dependent responses were also affected. We noticed that at low concentrations of Ag (≤2 molecules/μm²), overnight-cultured thymocytes appeared to form contacts more readily than fresh cells (Fig. 1E), suggesting that the up-regulation of TCR may also increase the sensitivity of contact formation. We therefore quantitated contact formation in response to varying densities of Ag. To do this, we determined the percentage of live cells forming contacts, by first counting the number of live cells in a microscopic field (Fig. 2A, left), then counting the number of those cells that formed IRM contacts above a minimum size (see Materials and Methods) (Fig. 2A, right). This method has the advantage that it accounts for possible variation in the number of cells present in each field examined. At high Ag density, both freshly isolated and overnight-cultured thymocytes readily adhered to the bilayer (∼80% of live cells) (Fig. 2B). With decreasing Ag density, however, overnight-cultured cells formed contacts more efficiently than fresh cells; the dose-response curve revealed an apparent increase in sensitivity to Ag of at least 100-fold after in vitro culture (Fig. 2B).

We considered a possible limitation of this method of quantitation. Because a portion of the thymocytes died during overnight culture, we equalized for live cell numbers in our calculations for the results shown in Fig. 2B. However, it is possible that fresh thymocyte cultures contained a mixture of Ag-responsive and Ag-unresponsive cells. During the overnight culture period, cell death may occur selectively in the Ag-unresponsive cell population. Therefore, the apparent increase in sensitivity to Ag, when expressed as a percentage of live cells (Fig. 2B), would simply be due to the selective survival of Ag-responsive cells. To address this possibility, we performed a separate experiment (Fig. 2C) in which we counted the absolute number of cell contacts per microscopic field remaining after unbound cells were washed away in the process of fixing and staining cells for immunofluorescence. In this experiment, we used a fixed volume of cells in culture media at each time point, without normalizing for the number of live cells remaining after in vitro culture. With this method, therefore, if 50% of cells died during overnight culture, then the input number of live cells would decrease by 50%, and an increase in the number of cell contacts could not be due to selective survival of cells able to respond to Ag. By this method, we again found an increase of sensitivity of thymocytes after overnight culture (Fig. 2C). The maximum number of contacts per field was variably decreased after overnight culture (Fig. 2C and data not shown), presumably reflecting the death of some of the cells in culture, but the shape of the dose-response curves was similar to that seen with the live cell assay (Fig. 2B), indicating that the sensitivity of contact formation was consistently increased after overnight culture.

Time course of enhancement of DP thymocyte responses

Taken together, our results indicate that the sensitivity of MTOC polarization and cell spreading to Ag is greatly enhanced after overnight culture of DP thymocytes, suggesting that cytoskeletal rearrangements induced by TCR engagement are enhanced with higher TCR expression. In previous studies, culturing DP thymocytes for 4–8 h led to an increase in TCR level, a decrease in ζ phosphorylation, and enhancement of calcium flux in response to CD3 cross-linking (4, 5, 23). Given the possible involvement of calcium signaling in MTOC polarization (21) and cell spreading (11, 27), these results suggested that we should see enhancement of cytoskeletal responses occurring after similar periods of culture. To determine the time course of sensitization to TCR stimulation, we cultured thymocytes for increasing intervals before adding them to bilayers. There was a modest increase in sensitivity after 2 h of incubation (data not shown), but by 6 h, the sensitivity for both contact formation (Fig. 2, B and C) and MTOC polarization (data not shown) had increased to almost the same extent as seen after overnight culture. These results paralleled the increase in TCR level on DP thymocytes, which was clearly evident at 6 h and continued with longer incubation times (Fig. 1C). After 6 h of
Cell spreading of thymocyte populations on Ab-coated surfaces

Our results suggest that releasing DP thymocytes from the thymic environment greatly enhances their ability to adhere and polarize in response to a TCR signal. The DP thymocytes we used in this study were taken from a “nonselecting” thymic environment, and hence represent newly developed DP thymocytes that have not received any positively or negatively selecting signals through the TCR. A possible limitation of this system is that transgenic TCRs are expressed earlier in development than naturally arising TCRs (28) and are expressed at higher levels on DP thymocytes than in nontransgenic mice; we therefore wished to confirm our observations using naturally arising thymocytes from nontransgenic mice. We also considered the possibility that the lipid bilayer may provide a relatively weak stimulation of the TCR, thus explaining the relatively poor responses of DP thymocytes to antigenic simulation in this system.

To address these issues, we used thymi from nontransgenic C57BL/6 mice, and supplied a polyclonal TCR stimulus by allowing the cells to adhere to glass coverslips coated with Abs to CD3, alone or in combination with other Abs. Previous studies have used similar systems to show that a signal through the TCR alone is sufficient to induce MTOC polarization (21) and actin-dependent cell spreading (11) in Jurkat T cells. This system offered the additional advantage of allowing us to directly compare the responses of DP thymocytes with naturally arising CD4+ or CD8+ SP thymocytes, which express higher levels of surface TCR than DP thymocytes, and would be expected to behave similarly to mature T cells. To distinguish these populations of thymocytes, we fixed the cells after incubation on Ab-coated surfaces and stained them with Abs to CD4 and CD8.

We found a dramatic difference between thymocyte populations plated on anti-CD3: CD4+ and CD8+ SP thymocytes spread and adhered efficiently, whereas DP thymocytes formed small, ill-defined IRM contacts (Fig. 3A, upper). We initially planned to examine cell spreading and MTOC polarization after 30 min of incubation, but found that while SP thymocytes formed robust contacts with the substrate, DP thymocytes formed such poor contacts that very few of these cells stayed adherent after fixation and staining. When we examined the time course of contact formation, we found that DP thymocytes formed small contacts that peaked in size in 10–15 min, then shrunk and became less defined (data not shown). We therefore used 15-min incubations for subsequent experiments, to maximize contact formation in DP thymocytes and allow us to judge MTOC polarization when cells were added to anti-CD3-coated surfaces (Fig. 3A).

To compare the sizes of the cell contacts among different populations, we traced the outlines of cell contacts from images of cells stained for CD4 and CD8 at the plane of the bilayer (i.e., Fig. 3A, right). Measuring the contact areas of DP and CD4+ SP thymocytes (≥120 cells of each type) confirmed our initial impression: DP thymocyte contacts were uniformly small, whereas CD4+ SP thymocyte contacts were more variable in size and the majority were larger, with a peak size about three times larger than DP thymocytes (Fig. 3B). It is possible that part of the difference in maximum contact size between the two populations may be attributable to a difference in cell size, as CD4+ SP thymocytes were slightly larger than DP thymocytes, as measured by forward scatter on FACS analysis (mean values, 481 vs 440). However, as discussed, contact formation by DP thymocytes was much weaker and more transient than by CD4+ SP thymocytes, and it is likely...
that lower TCR levels and weaker signaling through CD3 explain most of the difference in cell spreading. CD8⁺ SP thymocytes were not present in sufficient numbers to provide a reliable comparison, but their ability to spread on anti-CD3 appeared to be similar to CD4⁺ SP thymocytes (Fig. 3A and data not shown).

These results suggested that an isolated signal through CD3 was insufficient to induce cell spreading in DP thymocytes, consistent with poor signaling seen previously with CD3 cross-linking (5, 29). DP thymocytes nonetheless show sensitive responses to Ag presented by APCs (7), suggesting that additional adhesion and costimulatory molecules may contribute to signaling. We therefore asked whether adhesion of DP thymocytes could be facilitated by plating them on anti-CD3 together with Abs to additional cell surface molecules. When thymocytes were added to surfaces coated with Abs to CD3 and to the costimulatory molecule CD28, DP thymocytes formed contacts of similar size to those formed on anti-CD3 alone, but with better-defined contacts by IRM imaging, and more evident exclusion of CD4 and CD8 from the contact area, similar to SP thymocytes (Fig. 3A, middle). On Abs to CD3 and CD11a (the α-chain of LFA-1), DP and SP thymocytes formed round-edged, dark contacts by IRM imaging, and the size of the DP thymocyte contacts was larger than seen with anti-CD3 alone or with anti-CD28 (Fig. 3, A and C). Hence, Abs to accessory molecules on DP thymocytes facilitated their ability to spread on an anti-CD3-coated surface, whereas SP thymocytes spread efficiently on anti-CD3 alone, and their behavior was not altered by costimulatory signals (Fig. 3C).

When plated on either anti-CD28 or anti-CD11a alone, both DP and SP thymocytes readily formed contacts, but notably, CD4⁺ SP thymocyte contacts were much smaller than those formed on anti-CD3 (Fig. 3C). We also note that DP and CD4⁺ SP thymocytes formed contacts of similar size on anti-CD28, indicating that the difference in cell size we have noted does not necessarily correlate with dramatically larger cell contacts. Hence, the difference in cell spreading between DP and SP thymocytes is specific to TCR-CD3-mediated adhesion, and anti-CD3 is both necessary and sufficient for the dramatic cell spreading seen with SP thymocytes.

**MTOC polarization in response to immobilized anti-CD3**

We assessed the ability of DP and SP thymocytes to polarize their MTOC in response to anti-CD3 and other Abs, by visualizing microtubules within the area of contact with the substrate (Fig. 4A). MTOC polarization by DP thymocytes plated on anti-CD3 was inefficient, with <25% of those cells that formed a measurable contact having the MTOC visible within the contact area (Fig. 4B). This result was not surprising, because the cells formed weak contacts under these conditions, as described. However, when the cells were plated on anti-CD3 and anti-CD28 together, conditions that facilitate contact formation (Fig. 3A), MTOC polarization was still poor (Fig. 4, A and B). MTOC polarization on anti-CD11a alone or anti-CD3 and anti-CD11a was modestly increased, but still occurred in the minority of cells (Fig. 4, A and B). These results are consistent with our findings in the bilayer system, where efficient MTOC polarization in freshly isolated DP thymocytes was only seen at the highest concentration of Ag (Fig. 1A).

When we compared MTOC polarization between DP and SP thymocytes, we found that CD4⁺ SP thymocytes showed efficient MTOC polarization on anti-CD3 alone (>50%), and that this response was only slightly increased with the addition of anti-CD28 or anti-CD11a (Fig. 4B). Anti-CD3 was unique in its ability to induce MTOC polarization, as SP thymocytes did not polarize well in response to anti-CD28 or anti-CD11a alone (Fig. 4B). Hence, anti-CD3 appeared to deliver a unique signal that allowed CD4⁺ SP thymocytes to spread and undergo cytoskeletal polarization, and this response was deficient in DP thymocytes.
In vitro culture enhances responses of DP thymocytes to immobilized Abs

Given the dramatically increased sensitivity of DP thymocytes to polarization on lipid bilayers after culture, we asked whether the same treatment would affect cell spreading and MTOC polarization on Ab-coated surfaces (Fig. 5). Overnight culture of C57BL/6 thymocytes caused the up-regulation of TCR levels on DP cells, albeit to lower levels than on CD4+ or CD8+ SP thymocytes (data not shown). Cell spreading was facilitated: after overnight culture, many DP thymocytes plated on anti-CD3 formed dark, well-defined IRM contacts with the exclusion of CD4 and CD8 from the contact area, whereas very few freshly isolated DP thymocytes did so (Fig. 5A). The average contact size of DP thymocytes on anti-CD3 alone or with anti-CD28 was increased after overnight culture (Figs. 5B and 6), although the contacts were still smaller than those formed by CD4+ SP thymocytes (Fig. 6). Polarization of the microtubule cytoskeleton was also enhanced dramatically: after overnight culture, the majority of DP thymocytes displayed polarized MTOCs on anti-CD3 alone or with anti-CD28 (Fig. 5, A and B). The effect of overnight culture was less dramatic with DP thymocytes incubated on anti-CD3 with anti-CD11a, with only a mild increase in cell spreading and polarization (Fig. 5, B and C).

The enhanced cell spreading and MTOC polarization of DP thymocytes after overnight culture led us to ask whether culturing DP thymocytes would also affect actin polymerization. To address this question, we incubated DP thymocytes on anti-CD3 and anti-CD28, a combination of Abs that allowed contact formation (Fig. 3A) with either freshly isolated or overnight cultured cells. Using these Abs, both contact size (Fig. 5B) and MTOC polarization (Fig. 5C) were increased after overnight culture, suggesting that actin polymerization or polarization might also be affected. We localized F-actin by staining fixed cells with fluorescently labeled phallolidin, and compared the pattern and intensity of F-actin accumulation between freshly isolated and overnight cultured DP thymocytes (Fig. 7). Prominent staining of F-actin was seen at the periphery of the cell contacts in overnight-cultured DP thymocytes compared with freshly isolated cells (Fig. 7A). To determine the extent of polarization of F-actin toward the TCR stimulus, we obtained images of F-actin staining at increasing distances from the plane of contact (Fig. 7B). With freshly isolated DP thymocytes, many cells showed staining that appeared to be of similar intensity at increasing distances from the substrate (Fig. 7B, Cell 1), whereas with overnight cultured cells, the majority of cells showed most intense staining at the plane of contact (Fig. 7B, Cell 2).

To quantify actin polarization, we transformed these series of images (Z-stacks) to X-Z projections using IPLab imaging software, and used pseudocolored images to compare the relative intensity of staining at increasing distances from the cell substrate contact (Fig. 7C). We used three categories to rate the degree of polarization in individual cells: unpolarized, partially polarized, or polarized (see Materials and Methods for details). Representative cells from each category are shown in Fig. 7C; polarized cells showed the most intense staining at the level of the contact with the substrate. We found that overnight culture dramatically increased actin polarization: most of the freshly isolated cells were unpolarized or partially polarized, whereas the majority of overnight cultured cells were polarized (Fig. 7D; >50 cells analyzed for each condition).

In summary, our results show that overnight culture of DP thymocytes from C57BL/6 mice caused increased cell spreading and polarization of both the microtubule and actin cytoskeleton, and that this treatment enhanced contact formation and MTOC polarization of TCR transgenic DP thymocytes on lipid bilayers. The effect of in vitro culture in both experimental systems may be due to an increase in TCR level and therefore greater TCR signaling. However, cell spreading by both DP and CD4+ SP thymocytes was also enhanced after in vitro culture when the cells were plated on Abs to CD28 or CD11a alone (Figs. 5B and 6). The level of these two accessory molecules did not change after in vitro culture (data not shown), indicating that a more universal increase in cell adhesiveness and spreading, rather than a TCR-specific effect, may underlie our findings.

Discussion

In this report we have measured morphological responses of thymocytes to a TCR signal: cell spreading on an Ag-presenting surface and the polarization of the cytoskeleton toward the activating signal. These responses to TCR activation were inefficient in DP thymocytes and could be dramatically up-regulated by culturing the cells in vitro before adding them to lipid bilayers or Ab-coated surfaces. After culture, DP thymocytes behaved more similarly to SP thymocytes, suggesting that releasing DP cells from the thymic environment mimics some aspects of positive selection. It has been shown previously that culturing dispersed DP thymocytes for 4–8 h allowed TCR up-regulation and increased calcium flux upon TCR cross-linking (5, 23, 30). Our experiments extend these results to cytoskeleton-based cellular responses, and show that the
Ag sensitivity of these responses can increase at least 100-fold after culture.

Taken together, these results suggest that the intrathymic environment limits signaling via the TCR on DP thymocytes, through regulation of the level of TCR on the cell surface and biochemical modifications of the ζ-chain of TCR-CD3 complex. However, numerous studies have concluded that DP thymocytes are more sensitive to Ag than SP thymocytes or peripheral T cells (6, 7, 31–36). It seems contradictory, then, to propose that the thymus maintains DP thymocytes in a relatively insensitive state. However, as discussed below, the evidence for the increased sensitivity of DP thymocytes is mainly based on the quality of peptide-MHC complex presented (strength of ligand), rather than the numerical sensitivity to an agonist peptide (quantity of ligand). Our results suggest, therefore, that DP thymocytes are programmed to respond efficiently to weak ligands, and that the low level of TCR in the thymus may be a compensatory response to prevent excessive thymocyte deletion and fine-tune selection, as suggested previously (33).

There is abundant evidence that DP thymocytes show stronger relative responses to lower affinity peptides than do mature cells (6, 32–36). This pattern of reactivity provides a “biochemical margin of safety” in the deletion of self-reactive T cells and allows positive selection to occur through interactions with low affinity peptides (1, 3). In most published reports, however, the sensitivity to an agonist peptide was comparable between DP thymocytes and T cells or SP thymocytes (33, 35), and in fact DP thymocytes showed relatively insensitive CD69 up-regulation in response to anti-CD3 (33). Some studies comparing DP and SP thymocyte sensitivity were based on assays involving overnight incubation of dispersed thymocytes in vitro (6, 7). Using such an extended incubation could be misleading, because it would allow sufficient time for DP thymocytes to first up-regulate their TCR levels and then respond to low levels of Ag, yielding results that would not represent the true sensitivity of DP thymocytes in vivo. Our results suggest that although DP thymocytes show better relative reactivity to lower affinity peptides, their cytoskeletal responses to TCR stimulation are weak, and their sensitivity to an agonist peptide may be low. We should note that we have measured morphological responses to TCR stimulation, rather than cell death; additional experiments will be needed to determine whether our results can be extrapolated to the sensitivity of DP thymocytes to negative selection. An additional caveat to our results is that they reflect the behavior of mainly “preselection” DP thymocytes, and that TCR up-regulation after the delivery of a positively selecting signal may increase the sensitivity of DP thymocytes in vivo.

A simple explanation for our results is that DP thymocytes are insensitive to Ag because of low level of TCR. Studies in Jurkat (37) and dual-receptor transgenic T cells (38) have supported the

FIGURE 5. Overnight culture enhances contact formation and MTOC polarization in DP thymocytes on Ab-coated surfaces. A, freshly isolated thymocytes (top) or overnight-cultured thymocytes (bottom) from C57BL/6 mice were incubated on coverslips coated with mAbs to CD3 for 15 min, fixed, and stained for CD4 and CD8 (green and red; center) and tubulin (right). DP thymocytes showed stronger IRM contacts (left), larger contacts (center), and more polarized MTOCs (right) after overnight culture. Scale bar, 5 μm. B, mean contact sizes of freshly isolated and overnight-cultured DP thymocytes measured from images of cells incubated on the indicated combinations of Abs and stained for CD4 and CD8. C, MTOC polarization by freshly isolated or overnight-cultured DP thymocytes cultured on the indicated combinations of Abs and stained for tubulin. Contact size and MTOC polarization were assessed for at least 120 cells per condition.
idea that higher levels of receptor correlate with stronger responses to a TCR stimulus. The inhibition of DP responses in the thymus may therefore be due simply to lower TCR levels. If this is the
case, one would expect T cell development to be altered if the level of TCR on DP thymocytes were increased in vivo. Indeed, mice deficient in degranulation-promoting adapter protein ADAP (39) or c-Cbl (40) have increased levels of TCR-CD3 complex on DP thymocytes and show enhanced positive selection, although in these cases the effects on selection may be due to more general effects of these gene deletions on signal transduction rather than just an effect on the level of TCR.

The TCR-CD3 complex on DP thymocytes is not only expressed at lower levels, but also has biochemical modifications that could inhibit TCR signaling (and may determine cell surface expression of TCR). The ζ-chain of the TCR-CD3 complex, which is phosphorylated on ITAM motifs in mature T cells upon TCR cross-linking (41) is constitutively phosphorylated at high levels in DP thymocytes (4). ζ phosphorylation is Lck-dependent and results in constitutive association of inactive Zap70 with phospho-ζ (42, 43). These modifications may play a causative role in the low level of surface TCR on DP thymocytes because ζ phosphorylation is linked with TCR internalization in mature cells, and newly synthesized TCR-CD3 complexes in DP thymocytes are targeted for degradation (44). Constitutive Lck signaling, through interactions of CD4 or the TCR-CD3 complex with MHC class II expressed on thymic epithelium, may underlie ζ phosphorylation in DP thymocytes. Naïve T cells in the lymph node also have phospho-ζ at steady state, which is thought to maintain these cells in a higher state of activation (45), unlike the apparent case in DP thymocytes. Our experiments do not distinguish whether diminished responses in DP thymocytes is due to simply lower levels of TCR, or diminished signaling because of constitutive ζ phosphorylation.

An alternative explanation for our results is that TCR-mediated responses in DP thymocytes are more dependent on accessory signals than in SP thymocytes or mature T cells. DP deletion in vitro, which is highly sensitive to Ag presented on some types of APCs (7), appears to require a signal through CD28 to occur efficiently, both in Ab cross-linking studies (46) and experiments with APCs expressing a negatively selecting peptide (47). Our simplified systems may therefore lack accessory signals provided by the thymus that make up for weak signalizing through the TCR alone or with an additional signal only via CD28 or LFA-1. We note that adding Abs to CD28 or CD11a together with anti-CD3 facilitated DP thymocyte contact formation, and anti-CD3 with anti-CD11a caused a moderate increase in MTOC polarization (Fig. 5C). In the bilayer system, high densities of Ag in combination with ICAM-1 could induce efficient cell spreading and polarization (Figs. 1E and 2B), consistent with the efficient MTOC polarization noted in TCR thymocytes stimulated with high concentrations of Ag presented by splenic APCs (48). In our studies, the lipid bilayer system more closely mimics in vivo Ag presentation than do plate-bound Abs, because it uses physiological, mobile ligands for TCR and LFA-1, and allows the recruitment of Lck to the TCR via interactions of CD4 with peptide-MHC complex.

We studied cell spreading and MTOC polarization because we were interested in possible differences between immature and mature T cells in the translation of a TCR signal to cytoskeletal rearrangements. Cell spreading in response to a TCR signal represents a convenient model system for the formation of a contact area between a T cell and an APC. Previously, T cell lines have been shown to spread efficiently on anti-CD3 (9–11), and SP thymocytes behaved similarly in our system. By contrast, DP thymocytes formed small, transient contacts that were ill-defined on IRM (Fig. 3), but that became more robust after overnight incubation of the cells (Fig. 5). As TCR-induced spreading depends on signal-induced actin polymerization (11), our results suggest poor transfer of TCR ligation to cytoskeletal rearrangements in this cell type. Interestingly, phospho-ζ associates with the actin cytoskeleton upon TCR cross-linking in mature T cells but not DP thymocytes (49), providing a possible mechanistic basis for our findings.

MTOC polarization in T cells uses many of the same signaling pathways that lead to actin polymerization and cell spreading. The Rho family GTPase Cdc42 was implicated in MTOC polarization and actin accumulation at the site of T cell activation (50). Calcium signaling precedes MTOC polarization in Jurkat T cells plated on anti-CD3 (21) and has been implicated in halting T cell migration after TCR signaling (27). Zap70 is important for transducing a TCR signal to linker of activation of T cells and phospholipase Cγ and hence calcium flux, and is necessary for MTOC polarization in T cells (21, 51). We found a general correlation between cell spreading and MTOC polarization, suggesting common signaling pathways may be inefficient in DP thymocytes. In addition, the formation of a contact zone associated with actin polymerization may contribute to MTOC polarization, as interactions between actin and microtubules have been described in other systems (52), and the arrangement of microtubules at the synapse suggests lateral interactions with the cell cortex (53).

Although MTOC polarization plays a likely role in effector functions, its importance in T cell development, motility, and activation remains unclear. In other cell types, MTOC positioning may help determine the direction of cell movement (54). In T cells, both cell spreading and MTOC polarization are related to the ability of T cells to stop in response to a TCR signal. Microtubule integrity is important for maintenance of a T cell-APC contact (11, 55), suggesting it may help maintain the immunological synapse in vivo. The relatively poor polarization and cell spreading that we have observed in freshly isolated DP thymocytes may prevent sustained contact with APCs. This observation could be one way in which thymocytes integrate developmental signals over a period of days, compared with effector T cells, in which responses to Ag stimulation occur rapidly.

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Disclosures
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