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Immature CD4⁺CD8⁺ Thymocytes Do Not Polarize Lipid Rafts in Response to TCR-Mediated Signals¹

Peter J. R. Ebert, Josh F. Baker, and Jennifer A. Punt²

TCR-mediated stimulation induces activation and proliferation of mature T cells. When accompanied by signals through the costimulatory receptor CD28, TCR signals also result in the recruitment of cholesterol- and glycosphingolipid-rich membrane microdomains (lipid rafts), which are known to contain several molecules important for T cell signaling. Interestingly, immature CD4⁺CD8⁺ thymocytes respond to TCR/CD28 costimulation not by proliferating, but by dying. In this study, we report that, although CD4⁺CD8⁺ thymocytes polarize their actin cytoskeleton, they fail to recruit lipid rafts to the site of TCR/CD28 costimulation. We show that coupling of lipid raft mobilization to cytoskeletal reorganization can be mediated by phosphoinositide 3-kinase, and discuss the relevance of these findings to the interpretation of TCR signals by immature vs mature T cells. The Journal of Immunology, 2000, 165: 5435–5442.

Mature CD4⁺8⁻ or CD4⁺8⁺ (single-positive or SP³) T cells develop in the thymus from immature CD4⁺CD8⁺ (double-positive or DP) thymocyte precursors. Like their descendants, CD4⁺CD8⁺ thymocytes respond to TCR stimulation by activating a cascade of signaling events. However, the consequences of TCR stimulation differ markedly between immature and mature T cells. Whereas mature T cells respond to TCR signals by dividing, immature CD4⁺CD8⁺ thymocytes do not proliferate after engagement of TCR. Rather, depending on both the avidity of TCR engagement and the specific involvement of costimulatory or coactivating molecules, CD4⁺CD8⁺ thymocytes undergo either maturation (positive selection) or apoptosis (negative selection) (1, 2).

TCR signals, alone, are not sufficient to induce T cells to divide, differentiate, or die. Instead, they must be accompanied by second or costimulatory signals, of which CD28 is one example. CD28 has been found to act as a costimulatory signal in both mature and immature T cells. In mature cells, TCR/CD28 costimulation results in activation, sustained IL-2 production, and proliferation (3, 4). In immature CD4⁺CD8⁺ thymocytes, however, TCR/CD28 costimulation results in apoptosis (5–8). Although CD28 is not unique in its ability to cooperate with TCR to induce thymocyte death, in vivo (9, 10), it is a potent costimulator of TCR-induced apoptosis in vitro. Purified subpopulations of cells can be stimulated by discrete receptor/ligand interactions either 1) via cells designed to present MHC/Ag and the CD28 ligand B7, or 2) via immobilized mAbs specific for TCR and CD28 (5–8). Because TCR/CD28 costimulation results in such different outcomes in immature and mature T cells, in vitro stimulation can be a useful probe for developmental differences in TCR signaling.

Viola and colleagues have recently shown that in vitro TCR/CD28 costimulation of mature T cells induces the recruitment of cholesterol- and glycosphingolipid-enriched membrane microdomains (lipid rafts) to the site of stimulation (11). Lipid rafts are unique substructures in the plasma membrane enriched for a variety of signaling molecules, including GPI-linked proteins (12–14), lipid-modified proteins (15, 16), and specific lipid subsets, including phosphatidylinositol (17, 18).

In T cells, lipid rafts carry particularly precious signaling cargo, including the src kinases lck and fyn (19, 20), the adaptor molecule linker for activation of T cells (LAT) (21), and phosphatidylinositol-4,5-bisphosphate (PIP₂), a lipid substrate for PLC and phosphoinositide 3-kinase (PI3K) (22). By recruiting such key signaling molecules to the site of signal initiation, rafts have been shown to quantitatively enhance (11) and could qualitatively influence signaling routes initiated by TCR/CD28 costimulation.

Lipid raft polarization occurs concomitantly with reorganization of the actin cytoskeleton (23–26) and the establishment of an elaborate T cell/APC contact structure called the immunological synapse (25, 27, 28–30). During the interaction between APC and T cell, TCR complexes colocalize with lipid rafts (31–33) and the TCR ζ-chain associates with actin (34, 35). How lipid raft recruitment is coordinated with actin reorganization and receptor clustering is unclear.

Because lipid rafts have the potential to regulate signals, they are attractive targets for investigation into developmental differences in TCR signaling between immature and mature T cells. In the present study, we assess the ability of immature CD4⁺CD8⁺ thymocytes to recruit lipid rafts in response to TCR/CD28 costimulation. We show that raft polarization in mature T cells is actin dependent, and, although CD4⁺CD8⁺ thymocytes polarize actin efficiently, they do not polarize lipid rafts in response to TCR/CD28 costimulation. Furthermore, we offer evidence that PI3K activity coordinates polarization of lipid rafts with reorganization of the actin cytoskeleton in mature T cells, and discuss the potential relevance of these findings to developmental differences in immature vs mature T cell responses.
Materials and Methods

Reagents

Surfactant-free polystyrene beads were purchased from Interfacial Dynamics (Portland, OR), LY294002 and cytochalasin D were purchased from Calbiochem (San Diego, CA), and biotinylated cholera toxin B subunit from Sigma (St. Louis, MO). mAbs, anti-TCR-β (H57-597), anti-CD28 (37.51), anti-CD4 (GK1.5), and fluorescein (FITC)-conjugated anti-CD69 were purchased from PharMingen (San Diego, CA), and Alexa 488 streptavidin, Alexa 488 phallidin, and Alexa 488 goat anti-rabbit Ig were purchased from Molecular Probes (Eugene, OR).

Mice

C57BL/6 mice were purchased from Taconic Laboratories. The 4- to 8-wk-old mice were used as a source of CD4^+ CD8^+ thymocytes, and 8-wk- to 6-mo-old mice were used as a source of mature T cells.

Cell isolation

CD4^+ CD8^- thymocytes were purified by plating freshly isolated thymocyte suspensions onto anti-CD8 (83-12-5, 1 μg/ml)-coated petri dishes (6, 8). After 1 h at 4°C, nonadherent cells were washed away, and adherent cells were recovered and plated for a second time on anti-CD8 (83-12-5)-coated petri dishes. Recovered adherent cells were >95% CD4^+ CD8^-.

Mature T cells were enriched from spleen by plating isolated spleen suspensions on petri dishes coated with rat monoclonal anti-mouse IgG (200 μg/ml) to remove B cells. Nonadherent cells were recovered after 1 h at room temperature and contained >85% CD4^+ or CD8^- T cells.

Cells were either stimulated immediately after isolation or after 4-h culture at 37°C in 5% CO2 in complete medium (RPMI, 10% FCS, 2 mM l-glutamine, 1 mM penicillin/streptomycin, and 5 × 10^-3 2-ME).

Cell stimulation

Beads (10^7 beads/ml) were incubated with 1 μg/ml anti-H57, 5 μg/ml anti-CD28, and/or 1 μg/ml anti-CD4 in PBS for 1.5 h at 37°C, then washed three times in serum-containing medium before use. Beads were incubated at 37°C with cells at a ratio of 2 beads per cell and at a cell concentration of 2 × 10^5 cells/ml for 20 min (for evaluation of actin and lipid raft polarization) or 1 × 10^6 cells/ml for 5 h (for evaluation of CD69 up-regulation and cell/bead conjugation).

For immunofluorescence, cell/bead mixtures were fixed with 10-μm incubation with 3% paraformaldehyde, followed by addition of an excess of RPMI before staining. For flow cytometry, cell/bead mixtures were stained immediately after stimulation.

Drug treatment

Mature T cells were incubated in the presence of 10 μM cytochalasin D in 0.05% DMSO, or 50 mM Ly294002 in 0.05% DMSO for 10 min before stimulation and for the duration of stimulation. Control cells were treated with the vehicle, 0.05% DMSO, alone.

Cell staining

A total of 5 × 10^5 fixed or fresh cells was distributed in each well of a 96-well plate and washed three times with staining medium (1% BSA, 15 mM NaNO3 in balanced salt solution (HBSS)). For actin and GM1 staining, fixed cells (from 20-min stimulations) were stained for actin or GM1 by 40-min incubation with 10 μg/ml phallidin-Alexa 488, or 10 μg/ml biotinylated cholera toxin B subunit, respectively. Cells were washed three times in staining medium. For GM1 staining, cells were subsequently incubated for 30 min in the presence of 10 μg/ml avidin-Alexa 488, and then washed three times in staining medium. For CD69 staining, unfixed cells (after 5-h stimulations) were stained by 40-min incubation with 1 μg/ml FITC anti-CD69, then washed three times in staining medium. For LAT staining, fixed cells were washed three times in PBS containing 10% FBS, permeabilized with 0.1% Triton X-100 for 4 min at room temperature, washed, and incubated for an additional 45 min in 10% FBS. Cells were then incubated at room temperature for 45 min with 10 μg/ml rabbit anti-mouse LAT primary, washed three times, incubated for 45 min with 10 μg/ml Alexa 488-conjugated goat anti-rabbit IgG1, and washed again.

After staining, cells were either analyzed by flow cytometry or resuspended at roughly 1 × 10^5 cells/ml concentration and plated onto hydrophobically coated slides (Carlson Scientific, Peotone, IL) for visualization by immunofluorescence microscopy.

Microscopy

Stained cell/bead mixtures were visualized at ×1000 magnification using a Nikon Labophot immunofluorescence microscope, with an Omega Optical (Brattleboro, VT) XF68 NS332 filter to observe Alexa 488 fluorescence. Conjugates were identified under bright field before their pattern of fluorescence was assessed. Bright field and immunofluorescent images were recorded digitally using Adobe Photoshop 4.0 (Adobe Systems, Mountain View, CA) with an AV Macintosh computer. Unless stated otherwise, 20–30 cell/bead conjugates were counted and scored as either negative or positive for accumulation of Alexa 488 fluorescence intensity at the cell/bead interface.

Flow cytometry

Stained cell/bead mixtures were acquired on a Becton Dickinson (Mountain View, CA) FACScalibur and analyzed with CellQuest software. For CD69 and GM1 staining, live cells were identified based on forward scatter (FSC)/side scatter (SSC) profile and their FITC intensity measured.

Quantitation of percentage of cell/bead conjugates

Cell/bead conjugates can be distinguished by their FSC and SSC profile. Whereas T cells exhibited a high FSC and low SSC profile and hugged the x-axis, beads displayed a low FSC and high SSC profile and hugged the y-axis. In contrast, stable cell/bead conjugates appeared as a single event with both high FSC and high SSC intensities. Percentage of cell/bead conjugates was calculated by dividing the percentage of cells falling into the cell/bead conjugate gate by the total percentage of live cells.

Results

Coengagement of TCR and the costimulatory molecule, CD28, induces lipid raft aggregation in mature T cells (11). We wished to determine whether immature CD4^+ CD8^- thymocytes, which respond very differently to TCR/CD28 costimulation, also polarize lipid rafts.

Conditions for optimal acute stimulation of CD4^+ CD8^- thymocytes

Whereas freshly isolated mature murine T cells mobilize Ca^2+ (36) and up-regulate CD69 in response to TCR-mediated stimulation (Fig. 1a), freshly isolated CD4^+ CD8^- thymocytes do not (36) (Fig. 1b). The inability of CD4^+ CD8^- cells to respond to acute stimulation is thought to be attributable to the sequestration of lck by CD4, which is engaged in vivo by thymic MHC class II (37, 38). This defect in signaling can be readily corrected by either 1) preincubating cells in suspension, thereby disrupting TCR/class II interactions, allowing lck to redistribute, or 2) simultaneously co-engaging CD4 with TCR and CD28 to directly recruit lck to the membrane.

![FIGURE 1.](http://www.jimmunol.org/pk/7625_07_JJul25_2017.png)
complex (37, 38). Indeed, both mature and immature T cells will up-regulate CD69 in response to simultaneous TCR/CD28/CD4 engagement (Fig. 1, c and d) and after preincubation for 4 h (data not shown), indicating that these are optimal conditions for evaluating early activation events in immature T cells.

**TCR/CD28 costimulation does not induce lipid raft polarization in immature CD4⁺CD8⁻ thymocytes**

Using the stimulatory conditions that gave optimal TCR signaling in CD4⁺CD8⁺ cells, we compared the ability of mature T cells and immature thymocytes to polarize lipid rafts upon TCR-mediated costimulation. We stimulated cells with beads coated with the indicated Abs for 20 min and visualized lipid rafts with fluorochrome-conjugated cholera toxin B subunit, which binds the raft-resident ganglioside, GM1 (39). As expected, mature T cells exhibited efficient lipid raft capping in response to TCR/CD28 costimulation (Fig. 2, a and c). The majority (70–85%) of mature T cell/bead conjugates showed a distinct accumulation of fluorescence at the cell/bead interface after TCR/CD28 costimulation (Fig. 2, a and c), but not after TCR stimulation alone (data not shown). In contrast, immature CD4⁺CD8⁻ thymocytes did not polarize lipid rafts in response to TCR/CD28 costimulation (Fig. 2, b, d, and e), even when lck activity was restored after preincubation (Fig. 2d) or via recruitment of CD4 (Fig. 2e). Surface expression of GM1 was comparable between resting immature and mature T cell populations (Fig. 3), indicating that observed differences in GM1 distribution are not a consequence of differences in staining efficiency between mature SP and immature CD4⁺CD8⁻ populations.

To verify the indication that lipid raft components were not accumulating in response to TCR stimulation of immature CD4⁺CD8⁺ thymocytes, we stained stimulated cells for LAT, a lipid raft-resident adaptor molecule involved in TCR signaling (21, 40, 41) (Fig. 4). Consistent with our observations with GM1, TCR/CD28/CD4 coengagement induced LAT accumulation at the site of stimulation among mature SP T cells, but not immature CD4⁺CD8⁻ DP thymocytes. Again, despite their inability to polarize raft components, thymocytes responded to the stimuli by up-regulating CD69 (data not shown).

**Lipid raft polarization is actin mediated**

It was possible that the inability of immature CD4⁺CD8⁻ thymocytes to recruit lipid rafts reflected an inability to polarize the actin cytoskeleton in response to TCR stimulation. To confirm that lipid raft polarization was, indeed, actin dependent, we stimulated mature T cells in the presence of cytochalasin D, which both actively destabilizes existing actin microfilaments and prevents further actin polymerization (Fig. 5). As expected, in the presence of vehicle
alone, TCR/CD28 coengagement induced lipid raft polarization among the majority of mature T cell/bead conjugates (Fig. 5b). However, in the presence of cytochalasin D, only 19% of cell/bead conjugates exhibited lipid raft polarization (Fig. 5d). The few cells that did score positive for polarization tended to exhibited only a small, local accumulation of rafts (Fig. 5d, right panel). Cytochalasin D also significantly inhibited the formation of cell/bead conjugates, which are distinguished by their FSC and SSC profiles (Fig. 5, a vs c). These data indicate that both lipid raft recruitment and the formation of stable cell/bead conjugates require TCR-induced cytoskeleton reorganization.

**Immature T cells reorganize their actin cytoskeleton in response to TCR stimulation**

Given that lipid raft recruitment requires a functional actin cytoskeleton, we addressed the possibility that immature thymocytes fail to polarize lipid rafts because of their inability to reorganize actin in response to TCR stimulation. We compared patterns of actin organization after stimulation of mature and immature T cells by staining with phalloidin, which binds polymerized actin. As expected, mature T cells responded to acute TCR stimulation (in the presence or absence of CD28) with actin polymerization (42), indicating increased formation of lamellipodia and actin collars, which appeared to increase the area of cell/bead contact, resulting in establishment of a greater number of stable cell/bead conjugates (Fig. 6, a and c).

Significantly, immature CD4⁺CD8⁻ thymocytes were also capable of polarizing actin in response to TCR stimulation. As expected, acute TCR/CD28 stimulation, alone, did not induce actin polymerization and resulted in the establishment of only a low frequency of cell/bead conjugates (Fig. 6b). However, when stimulated through coengagement of TCR/CD28 and CD4, CD4⁺CD8⁻ thymocytes polarized actin, formed stable cell/bead conjugates, and exhibited the same morphologic changes seen in mature T cells (Fig. 6d).

**Lipid raft polarization is dependent on PI3K activity**

The inability of CD4⁺CD8⁻ thymocytes to mobilize lipid rafts, despite their ability to reorient their cytoskeleton, suggests that immature thymocytes are unable to couple lipid rafts with actin in response to TCR/CD28 costimulation. Because CD28 costimulation is required for raft movement, but not for TCR-driven actin polymerization (42), it seemed reasonable to suppose that signals downstream of CD28 may be responsible for coupling actin reorganization with lipid raft movement in mature T cells.

PI3K has been implicated as one downstream effector of CD28 (43). To determine whether PI3K were involved in lipid raft movement in mature T cells, we stimulated T cells in the presence of the PI3K inhibitor, LY294002 (44). CD69 up-regulation was abrogated in the presence of LY294002 within the 5-h time frame examined, indicating that the inhibitor abrogated efficient TCR signaling (Fig. 7, a and d). More importantly, the PI3K inhibitor also significantly reduced the frequency of cells exhibiting lipid raft polarization. Whereas lipid raft accumulation was evident in 70% of cell/bead conjugates formed in the absence of LY294002, lipid raft recruitment was observed in only 20% of cell/bead conjugates formed in the presence of the PI3K inhibitor (Fig. 7f). Any lipid raft capping that was observed in the presence of LY294002 was loose and disperse, indicating that even among the few positive events, polarization was not optimal (Fig. 7f).

However, despite their inability to polarize lipid rafts in the absence of PI3K activity, mature T cells retained their ability to reorganize their actin cytoskeleton. Cell/bead conjugate formation (Fig. 7, b and e) and phalloidin staining (data not shown) were comparable in stimulated mature T cells in the presence and absence of inhibitor, indicating that TCR-mediated actin polymerization did not require PI3K activity. Together, these observations suggest that PI3K mediates TCR/CD28-induced coupling of lipid rafts to actin cytoskeleton in mature T cells.
Discussion
In this study, we demonstrate a developmental difference in the ability of T lymphocytes to recruit lipid rafts to the site of TCR/CD28 costimulation and provide evidence for a mechanism underlying TCR-mediated raft polarization. Specifically, we show that immature CD4\(^4\)CD8\(^1\) thymocytes reorganize their actin cytoskeleton, but do not polarize lipid raft components (specifically, both the ganglioside GM1 and the adaptor protein LAT) in response to TCR/CD28 costimulation. In addition, we show that a functional actin cytoskeleton is required for lipid raft recruitment in mature T cells and implicate PI3K in mediating the coupling of lipid rafts with the actin cytoskeleton.

These data have several implications concerning the biology and developmental significance of lipid rafts. First, they indicate that lipid raft polarization is not absolutely required for TCR-mediated signaling. Despite the inability of TCR/CD28 costimulation to induce lipid raft mobilization in CD4\(^4\)CD8\(^1\) thymocytes, TCR/CD28 coengagement initiates robust signals that induce CD69 up-regulation.
and apoptosis (36). These observations raise the possibility that, rather than being an obligatory event in TCR signaling, lipid raft polarization may be one regulatory option available to a T cell.

Concomitant with the recruitment of lipid rafts is the formation of an immunological synapse (25, 27–30) that serves to segregate and concentrate signaling components at the interface between T cell and APC. While the synapse is thought to separate molecules based on their size and avidity for their ligands, lipid rafts allow the cell to sort signaling machinery based on its affinity for a unique lipid microenvironment. Thus, both processes may act to quantitatively enhance and sustain TCR signals, while at the same time each can regulate distinct subsets of signaling molecules that can be included in those signaling pathways. Our results indicate that immature CD4⁺CD8⁻ thymocytes cannot take full advantage of one regulatory option available to mature T cells.

These data also suggest a potential mechanism for raft movement in mature T cells. Our results confirm that TCR-mediated lipid raft polarization relies upon an intact actin cytoskeleton. Data showing that 1) actin polarization can be induced by TCR signals, alone (42), but 2) lipid raft recruitment requires costimulatory signals (11) imply that a costimulation-dependent process links rafts to the actin cytoskeleton. The results presented in this study indicate that PI3K activity mediates the establishment of this link. Based on these data, we propose one possible model for costimulation-dependent raft polarization, illustrated in Fig. 8a.

In this model, raft-resident PI3K substrates, phosphoinositides (e.g., PIP₂) (22), are converted to D3 phosphoinositides (e.g., phosphatidylinositol-3,4,5-phosphate (PIP₃)) by TCR/CD28-mediated PI3K activity (which may or may not be strictly associated with CD28). In parallel, TCR engagement initiates signals that recruit molecules containing pleckstrin homology (PH) domains, which are capable of binding D3 phosphoinositides (45, 46). PH domain/PIP₃ interactions then provide the physical link between the TCR/CD3 complex and lipid rafts. Lipid rafts and actin cytoskeleton may be indirectly associated via the TCR complex, which binds to actin via the TCR ζ-chain (35). Alternatively, lipid rafts and actin cytoskeleton could be more directly associated via a PH domain-containing actin-binding protein. Nonetheless, PI3K activity provides the means by which rafts are connected to actin and the TCR complex.

This model suggests that the uncoupling of lipid raft recruitment and actin polarization seen in immature CD4⁺CD8⁻ thymocytes could be due to their inability to efficiently stimulate a PI3K activity after TCR/CD28 costimulation, as illustrated in Fig. 8b. Interestingly, this possibility is supported by our previous observation that TCR/CD28 coengagement generates apoptotic signals in CD4⁺CD8⁺ thymocytes in a PI3K-independent manner (8).

It is important to recognize that CD28 is not the only costimulatory molecule that can cooperate with the TCR to induce lipid raft recruitment. TCR/CD48 and TCR/CD5 coengagement can also efficiently polarize lipid rafts in mature T cells (33, 47) (J.F.B., unpublished observations). Notably, consistent with our indications that PI3K is required for raft recruitment, CD5 engagement can activate PI3K activity (48). (Because CD48 is a raft-resident protein, TCR/CD48 coengagement may recruit raft in a more passive manner.) It is also important to note that the role of PI3K in costimulation of primary T cells, particularly its requirement for IL-2 production, is controversial (49, 50). While our data show that PI3K activity is required for lipid raft recruitment, our observation that signaling can take place in the

**FIGURE 8.** One model for costimulation-dependent lipid raft polarization. a, Mature T cells. TCR-associated and CD28-associated signals would be generated by coengagement of these receptors through interaction with MHC/peptide and B7 ligands on APCs. CD28-associated PI3K activation would alter the lipid environment of rafts by phosphorylating phosphoinositides at the D-3 position and converting, for instance, PIP₂ to PIP₃. TCR activation and resulting kinase activity and docking of adaptor molecules to phosphorylated residues of the TCR/CD3 complex would inspire the recruitment of molecules with PH domains, such as vav (46). PH domains would then provide a physical link between lipid rafts and TCR complexes, which interact directly with the actin cytoskeleton via TCR-ζ (34). Immature CD4⁺CD8⁻ thymocytes. Although TCR signals result in recruitment of PH domain-containing proteins and the polarization of actin by immature T cells, TCR/CD28 costimulation does not activate PI3K activity and does not convert PIP₂ to PIP₃ in lipid rafts. In the absence of the link between PIP₃ and recruited PH domain-containing proteins, lipid rafts do not connect with polarized TCR complexes. Thus, signals generated by TCR/CD28 costimulation are neither amplified nor accompanied by unique signaling cascades that could be initiated by raft-specific molecules.

...
absence of recruitment introduces the possibility that lipid raft recruitment is not strictly required for all aspects of T cell stimulation.

Interestingly, biochemical studies assessing the redistribution of signaling molecules into lipid rafts indicate that TCR/CD3 stimulation, alone, can recruit TCR components to the lipid raft fraction of both T cells and thymocytes (32, 33). In addition, very recent studies indicate that positive selection signals also result in recruitment of signaling molecules to the raft fraction in thymocytes (51, 52). These observations, combined with our immunofluorescence studies (in which TCR signals do not mediate lipid raft polarization in immature CD4+CD8+ thymocytes, and, in agreement with Viola et al. (11), costimulatory activity is required for raft polarization in mature T cells), raise the possibility that biochemical and immunofluorescence approaches can reveal different aspects of lipid raft involvement. It is possible, for instance, that TCR signals induce a reorganization of components, perhaps in raft microdomains, which may be below the level of detection using conventional immunofluorescence microscopy. It is also important to consider the influence of different cell preparation protocols on results. Because even small numbers of APCs can provide costimulatory signals, it may be particularly important to purify mature T cell subpopulations away from APC when examining the effect of TCR signals, alone. In addition, interpretation of data from unseparated thymocyte populations can be confounded by the robust activity of mature SP thymocytes, which represent at least 15% of an unseparated preparation. Nonetheless, the ability to detect important yet subtle changes in lipid raft components is an advantage of biochemical approaches, while the ability to examine gross changes in raft behavior at the single cell level is an advantage offered by immunofluorescence. Our immunofluorescence results demonstrate a clear difference in the extent to which TCR costimulation induces polarization of lipid raft components in mature SP T cells vs immature DP thymocytes.

Collectively, our findings raise the possibility that differences in the consequences of TCR-mediated costimulation in mature and immature T cells may be related to developmental differences in the ability to recruit lipid rafts in response to TCR signals. We speculate that without the ability to fully polarize rafts, CD4+CD8+ thymocytes may be denied access to specific signaling routes that confer survival advantages. Consequently, TCR-mediated costimulation may induce apoptotic signals that run unopposed in immature CD4+CD8+ thymocytes, but not in mature T cells.

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