Immature CD4⁺CD8⁺ Thymocytes Form a Multifocal Immunological Synapse with Sustained Tyrosine Phosphorylation

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Summary

The immunological synapse formed during mature T cell activation consists of a central cluster of TCR and MHC molecules surrounded by a ring of LFA-1 and ICAM-1. We examined synapse formation in thymocytes undergoing activation in a lipid bilayer system by following the movement of fluorescent MHC and ICAM-1 molecules. Immature CD4⁺CD8⁺ thymocytes formed a decentralized synapse with multiple foci of MHC accumulation corresponding to areas of exclusion of ICAM-1. The MHC clusters and ICAM-1 holes were mobile and transient and correlated with active and sustained signaling, as shown by staining with antibodies against phosphotyrosine and activated Lck. Our findings show that signaling in immature thymocytes can result from a novel, multifocal pattern of receptor accumulation.

Introduction

A recent emphasis in T cell biology has been on understanding the three-dimensional movement of cell-surface receptors, adhesion molecules, and signal transduction proteins during T cell activation. Focusing on the area of contact between a mature T cell and an antigen-presenting cell (APC) has revealed the immunological synapse, a structure in which T cell receptors and their ligands, peptide-MHC (pMHC) complexes, are concentrated in the center of the cell-cell contact, with a ring of adhesion molecules surrounding this central region (Grakoui et al., 1999; Monks et al., 1998). The initial engagement of TCR with pMHC has been found to take place at the periphery of the contact, followed by centripetal movement of small clusters of pMHC and TCR (Grakoui et al., 1999; Johnson et al., 2000; Lee et al., 2002) to form the larger, stable, central cluster, called the central supramolecular activation cluster (c-SMAC) (Monks et al., 1998). Conversely, the binding of LFA-1, a leukocyte integrin on T cells, to its ligand, ICAM-1, occurs initially in the central region of the contact, but is then excluded from the center and forms the peripheral adhesion ring, or p-SMAC (Grakoui et al., 1999; Monks et al., 1998). The mature synapse forms within 5–30 min and can persist for several hours (Grakoui et al., 1999; Lee et al., 2002).

While the immunological synapse is critical for T cell function, the details and implications of its formation are poorly understood. Recent studies have focused on the forces that drive synapse formation and the relationship of the synapse to T cell activation. Both cytoskeleton-dependent and -independent processes have been proposed to underlie the movement of molecules in the synapse. An active actin/myosin-based translocation of T cell-surface molecules toward the synapse has been observed (Wulffing and Davis, 1996), and the movement of CD43 away from the synapse is mediated by the actin cytoskeleton via ERM proteins (Allenspach et al., 2001; Delon et al., 2001). On the other hand, the lateral segregation of molecules in the synapse may be a direct result of differences in size of the proteins involved (Davis and van der Merwe, 1996; Shaw and Dustin, 1997; van der Merwe et al., 2000), and mathematical modeling suggests that the characteristic target-shaped structure of the synapse is due to a spontaneous self-assembly process (Qi et al., 2001). The relationship between synapse formation and T cell activation has also been controversial: initial reports suggested that the synapse is necessary for sustained signaling leading to activation (Grakoui et al., 1999; Monks et al., 1998), but a recent study found that many biochemical events associated with signaling occur before the mature synapse is formed (Lee et al., 2002).

Activation via the TCR is important not only for mature T cell activation but also for T cell development (Goldrath and Bevan, 1999; Sebzda et al., 1999). CD4⁺CD8⁺ (double-positive, DP) thymocytes undergo positive or negative selection as a result of TCR signaling, and the outcome depends on a number of variables, including the strength of the TCR signal (Ashton-Rickardt et al., 1994; Hogquist et al., 1994; Liu et al., 1998; Sebzda et al., 1999), the type of APC (Anderson et al., 1993, 1996), and the presence or absence of defined costimulatory signals (McKean et al., 2001; Page et al., 1993; Punt et al., 1994). While TCR signaling uses many of the same pathways in DP thymocytes and mature T cells, several proteins that play important roles in mature T cell activation appear to be dispensable for thymic selection, such as protein kinase C (PKC)–δ (Sun et al., 2000). Thymocytes also appear to have a lower threshold for activation than mature T cells (Davey et al., 1998; Grossman and Singer, 1996; Page et al., 1994; Peterson et al., 1999; Williams et al., 1998). These findings led us to ask whether the immunological synapse of DP thymocytes would differ from that seen in mature T cells.

To study receptor patterning during thymocyte activation, we used a model APC consisting of MHC-peptide complexes and ICAM-1 incorporated into a glass-suspended lipid bilayer. The bilayer proteins are covalently labeled with fluorophores, allowing real-time and high resolution imaging of protein movement (Grakoui et al., 1999). We found that DP thymocyte activation was accompanied by a different pattern of pMHC and ICAM-1...
accumulation than was seen with mature T cells under the same experimental conditions.

Results

To study synapse formation in thymocytes, we used cells from TCR transgenic mice and lipid bilayers containing GPI-anchored MHC and ICAM-1 molecules. We used transgenic TCRs that recognized peptides presented by two different MHC molecules: N3.L2, recognizing Hb64-76/I-E^d, and 3A9, which recognizes HEL 48-62/I-A^d. T cells from both of these transgenic mice have been used previously in the lipid bilayer system (Dustin et al., 1996; Grakoui et al., 1999). For a source of DP thymocytes, these TCRs were expressed in RAG-1-deficient mice of the H-2^b haplotype. Cells expressing the N3.L2 or 3A9 TCRs are not positively selected in a thymus expressing only H-2^b-encoded MHC molecules, and in the absence of RAG-1, endogenous TCR gene rearrangements do not occur. Hence, thymocyte development in TCR transgenic, H-2^b RAG-1-deficient mice does not proceed past the DP stage (Figure 1 and data not shown). TCR expression was low in DP thymocytes compared with single-positive (SP) thymocytes or splenic T cells, and the LFA-1 level was lowest in DP thymocytes, slightly higher in splenic T cells, and highest in SP thymocytes (Figure 1).

Activation and Differentiation of DP Thymocytes on Lipid Bilayers

In previous studies, the incubation of mature T cells on lipid bilayers containing pMHC complexes and ICAM-1 led to T cell proliferation (Grakoui et al., 1999). To address whether DP thymocytes would show functional responses in this system, we incubated N3.L2 or 3A9 DP thymocytes on lipid bilayers containing the appropriate pMHC complex and ICAM-1 and looked for several markers of activation. After 24 hr, DP thymocytes from 3A9 (Figure 2) and N3.L2 (data not shown) transgenic mice showed early markers of activation: the coordinated downregulation of CD4 and CD8 (“dulling;” Figure 2A, middle panel) and upregulation of CD69 (Figure 2B). These responses were antigen specific, as N3.L2 thymocytes responded to I-E^d loaded with agonist peptide but not an irrelevant peptide (data not shown), and 3A9 thymocytes responded to agonist pMHC complexes but not to an irrelevant MHC molecule (Figures 2A and 2B). CD69 upregulation and CD4/CD8 dulling were not seen with ICAM-1 alone, and a minimal response was seen with the same concentration of antigen in the absence of ICAM-1 (Figure 2B). We also saw similar but weaker responses with lower concentrations of antigen or when a weak agonist peptide was substituted for Hb64-76 (data not shown).

These results suggested that DP thymocytes were receiving a strong activating signal from antigen and ICAM-1 in the lipid bilayer; however, we found no evidence for increased cell death of DP thymocytes upon activation, by forward/side scatter profiles, or by Annexin V staining (data not shown). On the contrary, we found evidence for positive selection: after 48 hr of incubation of 3A9 DP thymocytes on lipid bilayers, a significant portion of the cells acquired the pattern of CD4 and CD8 expression characteristic of CD4^+ SP thymocytes (Figure 2A). This differentiation occurred on bilayers with specific pMHC and ICAM-1 but not with an irrelevant MHC (Figure 2A) or with ICAM-1 alone (data not shown). The SP thymocytes arising on lipid bilayers expressed high levels of CD69, CD5, and bcl-2, markers of positive selection, but did not upregulate TCR to levels found in SP thymocytes arising from positive selection in vivo (data not shown). Hence, lipid bilayers containing pMHC and ICAM-1 cause DP thymocyte activation and mediate early steps in positive selection but fail to induce negative selection.

Multifocal Synapses in DP Thymocytes

We asked whether the activation of DP thymocytes in the lipid bilayer system was accompanied by the formation of a mature immunological synapse. When 3A9 DP thymocytes were added to lipid bilayers containing I-A^d/HEL and ICAM-1, the cells formed close contacts with...
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ters of I-A^k could be seen with some cells, which correlated with the ICAM-1 holes (Figures 3A and 3C).

The multifocal pattern of ICAM-1 accumulation was not unique to cells expressing the 3A9 TCR, because when N3.L2 thymocytes were added to lipid bilayers containing ICAM-1 and I-E^k/Hb, the ICAM-1 pattern was indistinguishable from that seen with 3A9 (Figure 3B). We did not see accumulation of I-E^k within the contact area with N3.L2 thymocytes, consistent with the low level of TCR on DP thymocytes. Our ability to detect I-A^k but not I-E^k clusters may be due to a higher mobility of the former in the lipid bilayer or to intrinsic differences in TCR-pMHC interactions in the two systems. When 3A9 or N3.L2 DP thymocytes were added to lipid bilayers containing only ICAM-1, the cells did not form close contacts like those shown in the IRM image (Figure 3A), demonstrating the antigen dependence of adhesion and patterning.

A striking feature of receptor patterning in thymocytes was its dynamic nature. The ICAM-1 holes and MHC clusters migrated within the contact area over a period of minutes, while the general multifocal pattern was maintained. This mobility can be seen as a difference in the positions of I-A^k clusters in images of the same cell taken 2 min apart (Figure 3C). The ICAM-1 holes can be followed more closely in movies of a field of 3A9 DP thymocytes (see Supplemental Movie S1 at http://www.immunity.com/cgi/content/full/16/6/839/DC1) or a single N3.L2 SP thymocyte forming a multifocal pattern (see Supplemental Movie S2 at the above URL).

In contrast to these kinetic clusters in thymocyte synapses, the central I-E^k accumulation (c-SMAC) in mature T cells synapses appeared to be fairly static (Grakoui et al., 1999). Highlighting this difference, we observed that with those SP thymocytes that formed a centralized, mature synapse (see below), the central ICAM-1 hole/I-E^k cluster was persistent and fairly immobile, whereas the coexisting peripheral ICAM-1 holes moved from minute to minute (see Supplemental Movie S3 at the above URL).

**Phosphotyrosine Accumulation in Areas of pMHC Engagement**

We noted that the characteristic pattern of dispersed, mobile clusters of pMHC, correlating with ICAM-1 holes, was sustained over several hours, suggesting that there was ongoing signaling in those regions of the membrane. To examine this issue, we looked at the pattern of tyrosine phosphorylation in relation to ICAM-1 patterning at the thymocyte-lipid bilayer interface. N3.L2 DP thymocytes were fixed on lipid bilayers during activation, permeabilized, and stained with an anti-phosphotyrosine mAb. Phosphotyrosine (P-Tyr) staining was seen in a pattern that resembled MHC accumulation in the previous experiments, with a number of brightly stained spots within the cell contact corresponding to holes in the ICAM-1 fluorescence (Figure 4A). The ICAM-1 pattern was poorly preserved after fixation and permeabilization, but where ICAM-1 holes were visible, bright P-Tyr staining was consistently seen. The staining for P-Tyr was specific, inasmuch as staining of cells with an isotype control mAb yielded no fluorescence (Figure 4B). There was also P-Tyr staining around the

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**Figure 2. Activation of DP Thymocytes on Lipid Bilayers**

DP thymocytes from 3A9 H-2^b RAG-1^-/- mice were incubated on bilayers containing agonist pMHC complexes [I-A^k-HEL] and ICAM-1, an irrelevant MHC (I-E^k) and ICAM-1, I-A^k-HEL alone, or ICAM-1 alone. After 24 or 48 hr of incubation, cells were recovered from bilayers and analyzed by flow cytometry.

(A) CD4/CD8 staining of the starting population (top) and cells incubated for 24 hr (middle panels) or 48 hr (bottom panels) on agonist pMHC complexes with ICAM-1 (left panels) or irrelevant MHC with ICAM-1 (right panels). The percentage of live cells in each boxed region is shown. The same number of total (ungated) events was collected for each sample. The percentage of live lymphocytes, by forward/side scatter analysis, was 59% before stimulation, and after 48 hr of stimulation it was 47% and 26% in cells stimulated with I-A^k-HEL + ICAM-1 and I-E^k + ICAM-1, respectively.

(B) CD69 staining in cells incubated for 24 hr on bilayers with I-E^k + ICAM-1 (dotted line, curve 1), I-A^k-HEL alone (thin line, curve 2), I-A^k-HEL alone (thick line, curve 3), or I-A^k-HEL + ICAM-1 (shaded, curve 4).

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**Figure 3. ICAM-1 Holes and MHC Clusters**

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the bilayer (the dark area in IRM images, Figure 3A), and ICAM-1 and I-A^k accumulated within the contact area (Figure 3A). However, the pattern of these proteins was distinct from the target-shaped synapse seen in mature T cells: there were multiple, small (~1 μm) areas of exclusion of ICAM-1 (i.e., holes, Figures 3A–3C), and small clusters of I-A^k could be seen with some cells, which correlated with the ICAM-1 holes (Figures 3A and 3C).

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periphery of the cell contact (Figure 4A), and rim staining was seen at focal planes several microns above the lipid bilayer. Hence, P-Tyr staining appeared to be associated with the plasma membrane over most of the cell, but was excluded from the ICAM-1 area of the lipid bilayer contact region and was slightly concentrated in the TCR foci.

By its nature, the anti-P-Tyr antibody used here recognizes a wide variety of protein substrates; we were therefore interested in detecting signaling events that are more specifically associated with TCR-mediated activation. To do this, we used an antibody that recognizes the tyrosine kinase Lck phosphorylated on tyrosine at amino acid 394 and therefore detects the activated form of the enzyme (Holdorf et al., 2002). During thymocyte activation on bilayers, phosphorylated Lck (P-Lck) was detected in a pattern very similar to that of P-Tyr, with multiple, bright spots within the cell contact correlating to holes in the ICAM-1 fluorescence, as well as staining at the periphery of the contact (Figures 4C and 4D).

Previous studies have shown that tyrosine phosphorylation in mature T cells reaches a maximum in the first several minutes of antigen engagement. This early peak corresponds temporally to the nascent immunological synapse, in which antigen engagement occurs at the periphery or in dispersed clusters, before the formation of a c-SMAC (Lee et al., 2002). In our system, we saw no change in MHC and ICAM-1 patterning at any time point, and we were therefore interested in whether the level or pattern of tyrosine phosphorylation changed with time. We compared thymocytes that had been incubated for 2 min or 1 hr on lipid bilayers and saw no obvious difference in the pattern or intensity of staining for P-Tyr (Figure 4B). Hence, thymocyte activation in our bilayer system is associated with sustained tyrosine phosphorylation and the accumulation of activated Lck in areas of TCR-MHC-peptide engagement.

ICAM-1 and pMHC Patterning in SP Thymocytes
We depleted DP thymocytes from N3.L2 (H-2b; see Figure 1) thymi with anti-CD8-coated beads, leaving a population depleted of DP cells, with predominantly SP cells
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Figure 4. Multifocal Accumulation of Phosphotyrosine and Activated Lck

(A) DP thymocytes from N3.L2 H-2b RAG-1/-/- mice were incubated for 1 hr on lipid bilayers containing ICAM-1 and I-E^k loaded with agonist peptide, then fixed, permeabilized, and stained with an anti-P-Tyr mAb (4G10) and a fluorescently labeled secondary antibody. P-Tyr staining (green) correlates to areas of exclusion of ICAM-1 (red). Three representative cells are shown. Similar results were seen with DP thymocytes from 3A9 H-2^d RAG-1/-/- mice (data not shown). Scale bar, 5 μm.

(B) DP thymocytes were incubated on bilayers as in (A) for the indicated times, then stained with anti-P-Tyr or an isotope control mAb as indicated. Multiple foci of P-Tyr staining were evident in >80% of cells forming contacts with the bilayer, and the pattern and intensity of staining were similar after 2 min or 1 hr of incubation. Scale bar, 5 μm.

(C) DP thymocytes were incubated for 1 hr on bilayers, fixed and permeabilized as in (A), then stained with a polyclonal antibody to activated Lck (P-Lck), followed by a fluorescently labeled secondary antibody. Activated Lck staining (green) correlates to areas of exclusion of ICAM-1 (red).

(D) Cells were incubated for 1 hr on bilayers and stained as in (C). Multiple foci of activated Lck staining were evident in >80% of cells forming contacts with the bilayer.

and some CD4^-CD8^- double-negative (DN) thymocytes remaining. We added these cells to bilayers with ICAM-1 and I-E^k-agonist peptide complexes and examined the patterns of ICAM-1 and I-E^k accumulation. In many cells, there was a multifocal pattern of ICAM-1 and I-E^k accumulation resembling that seen with DP thymocytes, with multiple clusters of I-E^k that correlated with ICAM-1 holes (Figure 5A). A larger number of ICAM holes/MHC clusters were seen in the multifocal synapses of SP thymocytes compared with DP thymocytes, and the size of the contacts was generally larger in SP thymocytes. In some cells, there was a predominant central cluster of I-E^k, i.e., a c-SMAC (Figure 5A, bottom cell; Supplemental Movie S3 at http://www.immunity.com/cgi/content/full/16/6/839/DC1). The latter pattern resembled the centralized synapse of mature T cells, although the number of SP thymocytes forming centralized synapses was variable between experiments, and the synapses were rarely as well organized as those seen with mature T cells (naive splenocytes, Figure 5C). Centralized synapses were never seen with DP thymocytes, suggesting that the ability to form a mature synapse is acquired after positive selection.

As mentioned, our SP thymocyte preparation also included a minority of CD4^-CD8^- cells, and a portion of these DN thymocytes express high levels of the N3.L2 TCR (see Figure 1). To determine if DN thymocytes could also form synapses, cells from the SP/DN thymocyte preparation were added to lipid bilayers with pMHC and ICAM-1, and after synapse formation they were fixed and surface stained with an anti-CD4 antibody to distinguish SP (CD4^-) from DN (CD4^-) thymocytes. The majority of adherent cells showed bright membrane staining for CD4 (Figure 5A), confirming that these cells are SP thymocytes, but there were also adherent cells that failed to stain for CD4, i.e., DN thymocytes (Figure 5B). We validated the CD4 staining by showing that DP thymocytes showed CD4 staining comparable to SP thymocytes and that purified DN cells, obtained by depleting both CD8^- and CD4^- cells with antibody-coated beads, failed to stain for CD4 (data not shown).

The synapses of DN cells resembled those of SP cells, with multifocal accumulation of I-E^k. We suspect that these N3.L2 (H-2^b) DN thymocytes arise after positive selection, because in N3.L2 RAG^-/- H-2^d mice we did not see this population of CD4^-CD8^-, TCR^- thymocytes
Figure 5. ICAM-1 and MHC Patterning in CD4+ SP Thymocytes

(A and B) N3.L2 thymocytes were depleted of CD8-expressing cells (DP and CD8+ thymocytes) using anti-CD8 MACS beads. The resulting cell preparation was incubated on a lipid bilayer containing fluorescently labeled ICAM-1 (red) and I-Eκ (green) loaded with agonist peptide. After synapse formation, cells were fixed and surface stained using an anti-CD4 mAb (fourth panel) to distinguish SP thymocytes (A) from DN thymocytes (B). (A) shows SP thymocytes. In some cells, I-Eκ accumulated in multiple, small (∼1 μm) clusters (top cell), correlating to areas of exclusion of ICAM-1 within the contact area. In other cells, I-Eκ was predominantly associated with a larger, central cluster (bottom cell). Scale bar, 5 μm. In (B), multifocal synapses were seen in DN thymocytes, which represented ~20% of cells forming contacts with the lipid bilayer. Multifocal synapses were seen, a subset of which had relatively small, round contacts with 2–4 foci of MHC accumulation (lower cell). This pattern was only seen with DN cells.

(C) Mature T cells. N3.L2 splenocytes were added to a lipid bilayer of the same composition as shown in (A) and (B). by FACS (Figure 1), and, as mentioned above, we also did not see clusters of I-Eκ in synapses of thymocytes from the latter mice. These DN, TCRhi cells may undergo positive selection without passing through a DP stage, i.e., in the absence of CD4 (Liu et al., 1996), consistent with the fact that the TCR expressed in N3.L2 mice shows a minimal dependence on CD4 for activation (Vidal et al., 1999).

In the majority of naive N3.L2 T cells that had visible I-Eκ clusters, a centralized synapse was seen (Figure 5C), as shown previously for in vitro-activated T cells (Grakoui et al., 1999). However, there were also a number of cells that formed a stable, multifocal synapse indistinguishable from the multifocal synapses seen in SP thymocytes (data not shown); hence, the multifocal pattern is not unique to thymocytes. We have not compared the functional responses of SP thymocytes and naive splenocytes, so we do not know to what degree these cell types are activated in the bilayer system. We are interested in using this system to determine, on a single-cell basis, if formation of a mature synapse is associated with T cell activation.

Discussion

Here we have described a novel pattern of antigen receptor and adhesion molecule accumulation during DP thymocyte activation. TCR engagement resulted in the movement of pMHC complexes to multiple, small (∼1 μm in diameter) foci which also contained tyrosine phosphorylated proteins and activated Lck and corresponded to areas of exclusion of ICAM-1. The patterning shown here was robust: the characteristic ICAM-1 pattern was apparent in >90% of adherent cells, formed within the first minute of contact with the lipid bilayer, persisted for over 2 hr of continuous observation, and could be seen over a 100-fold range of antigen concentrations (data not shown). These decentralized synapses share several properties with the immunological synapse of mature T cells: TCR/pMHC and LFA-1/ICAM-1 accumulated in distinct domains within the T cell-APC contact, consistent with previous models of T cell activation (Davis and van der Merwe, 1996; Shaw and Dustin, 1997), and the areas of TCR-antigen engagement also contained proteins associated with TCR signaling, fitting the concept of supramolecular activation clusters (Monks et al., 1998). However, the overall organization of the thymocyte-APC contact shown here is different from that described for mature T cells. Instead of a stable, target-shaped, centralized synapse formed after several minutes of engagement, we saw a mobile, decentralized pattern which formed rapidly and did not change in its overall structure over several hours.

Stimulation of DP thymocytes in our bilayer system resulted in robust activation signals, judged by CD69 upregulation and CD4/8 dulling, followed by the initial
steps in positive selection. In many in vitro systems, stimulation with agonist peptide leads to efficient negative selection of DP thymocytes (Peterson et al., 1999; Swat et al., 1991; Vasquez et al., 1992). The model system used here presumably lacks secondary signals present on many APCs that would lead to negative selection (Page et al., 1993). Similar results were seen in experiments that used a thymic epithelial cell line as the APC: presentation of an agonist peptide on class II MHC to DP thymocytes led to positive selection, not cell death (Wang et al., 1998). ICAM-1 has been found to inhibit negative selection in vitro (Kishimoto et al., 1996; Lucas and Germain, 2000). It is possible, then, that lipid bilayers incorporating B7 instead of ICAM-1 would induce negative selection in DP thymocytes. Further work incorporating additional ligands in the lipid bilayer will be necessary to determine if a different synaptic pattern is formed under conditions that lead to negative selection.

Our results show that DP thymocytes and mature T cells can form distinct receptor patterns when examined in a model APC system using a simplified stimulus (pMHC and ICAM-1). The mature immunological synapse seen in the lipid bilayer system (Figure 5C) has the same overall structure as synapses in cell-cell conjugates, using antigenic peptides presented by B cell tumor lines (Monks et al., 1998) or splenic APCs (Lee et al., 2002). In fact, the inverted, nascent synapse that was first seen using the lipid bilayer system (Grakoui et al., 1999) has recently been demonstrated in a cell conjugate system (Lee et al., 2002). Hence, this experimental system accurately predicted results seen with mature T cells and professional APCs.

The lipid bilayer system offers several potential advantages over cell conjugate systems: (1) real-time analysis of synapse formation; (2) unambiguous interpretation of patterns, because the movement of bilayer proteins reflects engagement by T cell proteins and is not subject to other forces that might affect the patterning of cell-surface molecules on T cells or APCs; and (3) high resolution imaging, in part because the model APC surface is confined to a single plane of focus. The relatively small, closely spaced (1–3 μm apart) ICAM-1 clusters of pMHC, which were visible in our system (Figure 3), might not be evident in the reconstructions of cell-cell interfaces that are necessary when conjugate systems are used.

Despite the advantages of the lipid bilayer system, its simplified nature means that the patterns of receptors shown here may differ from those formed in cell-cell conjugates. Our system lacks some costimulatory interactions known to affect thymocyte selection (Kishimoto et al., 1996; Lucas and Germain, 2000; Punt et al., 1994), although it is worth noting that CD28-B7 interactions increase naive T cell activation without influencing either the pattern of the synapse or the amount of accumulation of pMHC or ICAM-1 (Bromley et al., 2001). Other possible contributions from the APC, such as cytoskeletal interactions with receptors or formation of membrane microdomains, may influence synapse patterns during thymocyte selection.

While our report is the first description of persistent, small clusters of TCR/MHC, this pattern does resemble early stages of mature T cell activation. Previous studies in the lipid bilayer system (Grakoui et al., 1999; Johnson et al., 2000) showed the movement of small clusters of MHC and TCR to the middle of the contact, combining to form the c-SMAC. Similarly, in a cell-cell system, small clusters of TCR-associated CD3ζ-GFP were seen at early time points, coalescing within 15 min to form a larger central cluster (Krummel et al., 2000). Hence, the small MHC clusters shown here may be equivalent to structures formed initially in mature T cells, either dispersed or in a ring at the periphery of the cell, which then move centrally to form a larger, stable metacluster. These results suggest that early TCR clustering and signaling events occur similarly in mature T cells and DP thymocytes, but the subsequent capping of TCR seen in mature T cells fails to occur in thymocytes. The inability of DP thymocytes to form a centralized synapse in this system may be related to receptor levels: DP thymocytes have very low levels of TCR and somewhat lower levels of LFA-1 compared with mature T cells (Figure 1). However, SP thymocytes, with equal levels of TCR and higher levels of LFA-1 compared with mature T cells, had less of a tendency to make a mature synapse, with a smaller number of cells forming a central cluster, and smaller c-SMACs (Figure 5). Other aspects of signaling, membrane dynamics, and receptor binding may explain the differences in patterning seen here.

The small MHC clusters in multifocal synapses correlate with regions of robust staining with antibodies against P-Tyr or activated Lck. The level of P-Tyr staining in these membrane regions was comparable after 2 min or 1 hr of incubation, in contrast to mature T cells, in which an initial peak of phosphorylation is followed by TCR capping and the apparent attenuation of biochemical signaling at the T cell-APC interface (Lee et al., 2002). These observations highlight the resemblance of thymocyte synapses to early mature T cell synapses and suggest that immature thymocytes may be activated though a mechanism that involves sustained biochemical signaling. Interestingly, freshly isolated DP thymocytes have been shown to have constitutively phosphorylated TCRζ (Kearse et al., 1993; Nakayama et al., 1989), and Lck regulates this phosphorylation (van Oers et al., 1996). The tyrosine phosphorylation and activated Lck that we see associated with regions of TCR/pMHC clustering may therefore reflect the maintenance of preexisting phosphorylated proteins rather than de novo signaling. It should be noted that our experiments focused on the pattern of phosphorylation at the thymocyte-lipid bilayer interface and not the total level of phosphotyrosine in the cell. Further experiments will be needed to compare the kinetics of total cellular tyrosine phosphorylation in the lipid bilayer system with previously described models of thymocyte activation such as TCR crosslinking.

A characteristic of immunological synapse formation by mature T cells is that it halts migration (Dustin et al., 1997a). The cell stops apparently as a result of a switch in the direction of cortical actin movement, which flows toward the rear of a migrating cell but is redirected toward the APC upon synapse formation (Breitscher, 1996; Dustin and Cooper, 2000; Wulfing and Davis, 1998). While thymocytes are not highly motile on the pattern of phosphorylation at the thymocyte-lipid bilayer interface and not the total level of phosphotyrosine in the cell. Further experiments will be needed to compare the kinetics of total cellular tyrosine phosphorylation in the lipid bilayer system with previously described models of thymocyte activation such as TCR crosslinking.
cues. In thymocytes, positive selection in the deep cortical region is thought to trigger migration to the medulla, where the thymocyte may undergo negative selection or develop into a mature T cell (Prockop and Petrie, 2000). This migration is thought to involve the TCR-mediated sensitization of the thymocytes to chemokines in the medulla (Suzuki et al., 1999). Thus, a possible feature of the more dynamic thymocyte synapse is that it may not interfere with migration. A dynamic mode of mature T cell stimulation has been reported in collagen extracellular matrix where T cells migrate rapidly over the surface and between different dendritic cells (Gunzer et al., 2000). While the signals that trigger this behavior are not known, the multifocal immunological synapse may provide a model for T cell activation by serial encounters with APC.

It is known that chronic or repeated strong T cell stimulation can lead to antigen-induced cell death even in mature T cells (Green and Scott, 1994). Thus, the canonical immunological synapse of mature T cells may offer some protection from antigen-induced cell death by downregulating TCR signaling strength while maintaining a connection with the APC. The multifocal immunological synapse of thymocytes may be tuned to allow sustained TCR signals that may be necessary for sensitive selection events during T cell development.

Experimental Procedures

Mice

Mice expressing the transgenic TCR 3A9, recognizing I-Ak and hen egg lysozyme peptide HEL48-62 (Ho et al., 1994), were crossed from a B6.AKR (H-2b) background to the H-2a, Rag-1-deficient strain C57BL/6J-Rag1tm1Mom (The Jackson Laboratory, Bar Harbor, ME). N3.L2 mice were transgenic for the 3L2 TCR (Evavold et al., 1992). The previously described 3L2 mice (Grakoui et al., 1999; Kersh et al., 1998a) were made with a transgene that had a single base error in the Jβ segment resulting in an amino acid change at position 115 from threonine to alanine. The N3.L2 mice used here had the corrected 3L2 TCR sequence and were made as previously described (Kersh et al., 1998a). The soluble 3L2 TCR used in previous BALCore studies (Kersh et al., 1998b) was identical in sequence to the TCR used in N3.L2 mice described here. We found that the reactivity of N3.L2 T cells to a panel of altered peptide ligands (APLs) based on the Hb-97 antigen peptide was identical to that previously described (Grakoui et al., 1999) for T cells from 3L2 mice (D. Donnemeyer and P. M.A., unpublished observations). We also found that negative and positive selection in response to the same panel of APLs was the same for N3.L2 as previously described for 3L2 (Williams et al., 1999). Thus, the antigen reactivity of cells from N3.L2 and 3L2 mice was the same at all stages of T cell development. The only effect of the mutation in the TCR of 3L2 mice was to inhibit the DN (CD4+CD8−) to DP (CD4+CD8+) thymocyte transition, such that 3L2 T cells expressed a second TCR chain and did not develop in Rag-1-deficient mice, whereas N3.L2 TCR transgenic T cells developed normally in the absence of Rag-1. N3.L2 mice on a B6.AKR (H-2b) background were used as a source of T cells and isolated SP (CD4+CD8−) and DN thymocytes. For a source of N3.L2 DP thymocytes, we used N3.L2, Rag-1-deficient, H-2b− mice bred as described above for 3A9. In both N3.L2 and 3A9 Rag-1-deficient H-2b− mice, thymocyte development appeared normal up to the DP stage, with total thymus cellularity of ~10^6 in 6- to 8-week-old mice, but development did not proceed past the DP stage (see Figure 1).

Cell Preparation

Single-cell preparations of thymocytes were made from thymi of 4- to 12-week-old mice. CD8+ thymocytes were depleted from whole thymocyte preparations using MACS MicroBeads (Miltenyi Biotec, Auburn, CA). Freshly isolated splenocytes were used as a source of mature T cells.

Lipid Bilayers and Microscopy

GPI-anchored I-Eδ and ICAM-1 were prepared and incorporated into lipid bilayers as described (Grakoui et al., 1999). A GPI-anchored I-Aα molecule with covalently tethered hen egg lysozyme peptide (Fremont et al., 1998) was purified from transfected CHO cells (gift of E. Unanue) through the same procedure. I-Eδ and I-Aα-HEL were covalently labeled with Oregon Green reactive dye (succinimidyl ester; Molecular Probes, Eugene, OR), and ICAM-1 was labeled with Cy5-NHS (Amersham, Piscataway, NJ). Bilayer proteins were incorporated into egg phosphatidylcholine vesicles, and lipid bilayers were formed by incubating mixtures of these vesicles on clean glass in a parallel plate flow chamber (Bioptechs, Butler, PA) heated to 37°C. Cells were added to bilayers at 37°C in HEPES-buffered saline (pH 7.4) with 1% human serum albumin (HBS/HS.A). Images of cells (transmitted light), contact areas (interference microscopy), and accumulation of fluorescent bilayer proteins (epi-fluorescent illumination) were obtained as described (Dustin et al., 1997b) on an inverted microscope (Yona Microscopes, Silver Spring, MD) using the appropriate filter sets for fluorescent images. Fluorescent and IRM images were flat-field background corrected (Dustin et al., 1997b). Images of single cells shown here were further processed by deconvolution. In these supported bilayer studies, all fluorophore is restricted to a single plane. Therefore, all of the "blur" inherent in the optics is due to the two-dimensional point-spread function. The supported bilayer is a unique experimental setup that allows a simple and exact correction of the images for the point-spread function. Images were deconvolved for the two-dimensional point-spread function using a Wiener filter algorithm based on the two-dimensional Fourier transform capabilities of IPLab (Scanalytics Inc., Fairfax, VA). The point-spread functions were determined by imaging subresolvable beads and are sufficiently space invariant to be used at any region of the image.

Antibodies and Immunofluorescence

PE-anti-CD4, CyChrome-anti-CD8, and FITC-anti-CD69 monoclonal antibodies (BD Pharmingen, San Diego, CA) were used for FACS analysis. The mouse anti-phosphotyrosine mAb 4G10 was from Upstate Biotechnology (Lake Placid, NY). An isotope control antibody for 4G10 (C48-4, mouse IgG2b kappa) was from Pharmingen. A rabbit polyclonal antibody against an Lck peptide phosphorylated at tyrosine 394 (activated Lck) was affinity purified against the phosphorylated peptide used as the immunogen and subsequently absorbed against a nonphosphorylated version of the same peptide (Holdorf et al., 2002). Secondary antibodies used for immunofluorescence were goat anti-mouse Alexa Fluor 546 (Molecular Probes, Eugene, OR) and donkey anti-rabbit Cy3 (Jackson Immunoresearch, West Grove, PA). For intracellular staining, cells were fixed on lipid bilayers in 2% paraformaldehyde for 10 min, permeabilized briefly in 0.05% Triton X-100, blocked for 20 min in 5% nonfat dry milk, and incubated with primary and secondary antibodies for 30 min each. All steps were performed in flow chambers at 37°C with reagents diluted in HBS/HS.A with washes of 5 ml of HBS/HS.A between steps. 4G10 was used at 2 μg/ml, anti-Lck at 7 μg/ml, and secondary antibodies at 4 μg/ml. Images of P-Tyr and Lck staining were obtained using the same plane of focus as the lipid bilayer. For surface staining of CD4, cells were fixed in 4% paraformaldehyde for 15 min, blocked for 20 min in 5% nonfat dry milk, and stained with biotinylated anti-CD4, clone RM4-5 (BD Pharmingen), at 2.5 μg/ml, followed by streptavidin-Alexa546 (Molecular Probes) at 2 μg/ml, both for 30 min at room temperature in PBS.

Thymocyte Activation Assays

Lipid bilayers were formed on 15 mm glass coverslips in 24-well plates. I-Eδ was loaded with peptide for 24 hr in pH 5 buffer as described above. The bilayers were then washed, and thymocytes (1.5 × 10^6 per well) were added in RPMI with 10% fetal calf serum. Thymocytes were incubated on bilayers for 24 or 48 hr at 37°C in a tissue culture incubator. At the end of the incubation, thymocytes were recovered by pipetting, washed in FACS buffer (PBS with 0.5% BSA and 0.1% sodium azide), stained on ice with anti-CD4, anti-CD8, anti-CD69, anti-IgM, or anti-Lck, and analyzed by flow cytometry.
anti-CD8, and anti-CD69 antibodies, and analyzed by flow cytometry.

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