

Imaging Synapse Formation during Thymocyte Selection: Inability of CD3 ζ to Form a Stable Central Accumulation during Negative Selection

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Summary

TCR signaling can result in cell fates ranging from activation to tolerance to apoptosis. Organization of molecules in an “immunological synapse” between mature T cells and APCs correlates with the strength of TCR signaling. To investigate synapse formation during thymic selection, we have established a reaggregate system in which molecular recruitment of GFP fusion proteins to thymocyte:stromal cell interfaces can be visualized in real time. We demonstrate that negative selection is associated with efficient conjugate formation and rapid recruitment of p56^{lck} and CD3 ζ to an immunological synapse. Interestingly, CD3 ζ -GFP does not accumulate at the center of the synapse, as in mature T cells, but at the periphery across a wide range of ligand densities. This implicates differences in synapse geometry in initiation of alternate signals downstream of the TCR.

Introduction

Thymocytes undergo three stringent selection processes in the thymus before being exported to the periphery: T cell receptor (TCR) β -selection, positive selection, and negative selection. The latter two selection events involve interactions of nascent TCRs with MHC:peptide complexes on thymic antigen presenting cells (APCs). Positive selection results in maturation of T cells capable of recognizing foreign peptides in the context of self-major histocompatibility molecules (MHCs), while negative selection results in apoptosis of autoreactive thymocytes. The mechanism by which endogenous ligand encounters translate into positive versus negative selection remains an open question (as reviewed by Lo et al., 1986; Kisielow and von Boehmer, 1990; Janeway et al., 1992; Goldrath and Bevan, 1999; Hogquist, 2001; Alberola-Ila et al., 1997).

Following TCR engagement, the src-family tyrosine kinase lck is recruited to the TCR:CD3 complex, where it phosphorylates immune-receptor tyrosine-based activation motifs (ITAMs) on CD3 chains, initiating signaling cascades downstream of the TCR (Molina et al., 1992; Straus and Weiss, 1992; Wallace et al., 1995). CD4 and CD8 are noncovalently associated with lck (Turner et al., 1990) and act to strengthen TCR signals by recruiting lck to the TCR (Owens et al., 1987). It has recently been demonstrated

that a mutant form of lck incapable of interacting with CD4 and CD8 is unable to promote negative selection (Trobridge et al., 2001). Given that so many of the initial players in T cell activation and thymocyte positive and negative selection are the same, the question arises as to how the interaction of a TCR with a peptide:self-MHC complex could initiate a different program of events that lead to positive selection, negative selection, or mature T cell activation.

It is possible that the relative localization of these membrane-proximal signaling molecules during thymic selection and mature T cell activation could initiate alternative signaling cascades. Recent data have demonstrated that mature CD4⁺ T cell recognition involves the formation of an “immunological synapse” at the T cell:APC interface, exhibiting a complex spatial distribution of cell surface molecules. Specifically, TCR/CD3 and MHC molecules accumulate in the center of the synapse, while CD4 and the adhesion molecules LFA-1 and ICAM are recruited to the synapse periphery (Monks et al., 1998; Wulfing et al., 1998; Grakoui et al., 1999; Krummel et al., 2000). Recent work from our laboratory has demonstrated that accumulation of CD3 ζ and CD4 are rapid events, occurring within minutes of T cell:APC contact (Krummel et al., 2000). This temporal and spatial map of the immunological synapse provides a template against which we may compare the recruitment of signaling molecules in thymocytes undergoing selection. To date, studies on synapse formation have been based on cell interactions observed in monolayer cultures, raising the question as to whether similar events might occur within the three-dimensional (3D) architecture of lymphoid organs. Indeed, one study has suggested that conjugate formation can occur much more rapidly during transient interactions within collagen gels as compared to monolayers (Gunzer et al., 2000). As of yet, no real-time studies on living cells within lymphoid organs have been reported, but a recent immunohistological study on fixed tissue provides evidence of synapse formation within lymph nodes (Reichert et al., 2001).

Our aim has been to establish a system in which molecular recruitment to the thymocyte:stromal cell interface can be visualized in real time in living thymocytes within a three-dimensional thymic stromal environment capable of mediating positive and negative selection. We have expressed green fluorescent protein (GFP) fusions of lck and CD3 ζ in 5C.C7 TCR transgenic CD4⁺CD8⁺ (double-positive, DP) thymocytes using retroviral-mediated transduction. Transduced DP thymocytes from a nonselecting thymus (C57BL/6) were mixed with selecting (B10.BR) thymic stromal cells to generate a reaggregate capable of positively selecting DP to CD4⁺ SP cells and negatively selecting DP cells following addition of the agonist peptide moth cytochrome c 88-103 (MCC). High speed time-lapse microscopy was employed to visualize temporal and spatial recruitment of lck-GFP and CD3 ζ -GFP to the thymocyte:stromal cell interface.

Our results show that agonist peptide induces rapid clustering of lck and CD3 ζ at the thymocyte:stromal cell interface. Moreover, this interaction is sustained and the functional outcome is negative selection. By making 3D

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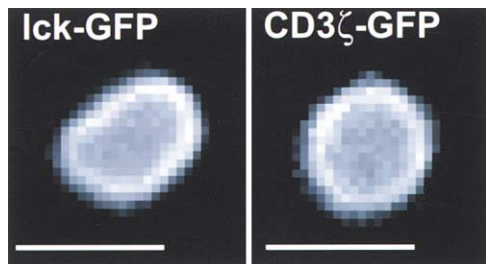


Figure 1. Lck-GFP and CD3 ζ -GFP Localize to the Plasma Membrane of Transduced DP Thymocytes

5C.C7 TCR transgenic C57BL/6 thymocytes were transduced with Lck-GFP or CD3 ζ -GFP retroviruses. As expected, both fusion proteins localized to the plasma membrane. Scale bar, 10 μ m.

reconstructions of the thymocyte:APC interfaces, we have been able to assess the patterns of Lck and CD3 ζ accumulations and show that the pattern of CD3 ζ at thymocyte synapses differs from that in mature T cells. Given that many key signaling molecules in negative selection of thymocytes and activation of mature T cells are the same, and yet the functional outcome varies between apoptosis and proliferation, it seems likely that different spatial organization of molecules like CD3 ζ at immunological synapses could contribute to the establishment of distinct signaling cascades. The system that we have established is generally applicable to visualizing the molecular machinery of many other interesting cellular interactions and processes that occur within the thymus.

Results

Efficient Transduction and Selection of DP 5C.C7 Thymocytes

Using retroviruses encoding Lck-GFP or CD3 ζ -GFP, we transduced 5C.C7 TCR transgenic DP thymocytes. Epifluorescent microscopy revealed that both fusion proteins localize to the plasma membrane, as do wild-type proteins (Figure 1). To obtain immature thymocytes that had not yet experienced selection, we transduced 5C.C7 TCR transgenic thymocytes from I-E^k-negative C57BL/6 mice. Since the 5C.C7 TCR requires I-E^k for positive selection (Fink et al., 1986; Fazekas de St. Groth et al., 1993), 5C.C7 transgenic thymocytes on a C57BL/6 background are predominantly CD4⁻CD8⁻ (double-negative, DN) and DP, with few mature CD4 or CD8 single-positive (CD4/8 SP) cells (data not shown). To determine the transduction level, thymocytes were stained with anti-CD8 (clone 53-6.7; Pharmingen) 24 hr after transduction to distinguish between DN and DP cells and analyzed by FACS to determine the percentage of GFP⁺ cells (Figure 2A). 28%–32% of DP thymocytes and 38%–42% of DN thymocytes expressed the fusion proteins.

To obtain transduced thymocytes at a developmental stage capable of undergoing selection, we enriched for DP thymocytes 24 hr after transduction by positively selecting on anti-CD8 coated beads. DP thymocytes were reaggregated with 2-deoxyguanosine (2-dGuo)-treated stroma from embryonic day 15 B10.BR mice with or without 1 μ M MCC(88-103) to generate stromal environments that mediate negative or positive selection, respectively.

After 2-dGuo treatment, which removes lymphoid and dendritic cells, and anti-CD45 bead depletion, which removes macrophages, over 80% of the stromal cells are epithelial and the rest are fibroblasts (Jenkinson et al., 1992). To show that negative selection could occur in these reagggregates, they were dissociated and stained for CD4 and CD8 expression after 72 hr in culture. Figure 2B demonstrates that in the absence of exogenous peptide B10.BR stroma positively select DP cells, yielding CD4 SP thymocytes. However, with 1 μ M of the agonist MCC(88-103), DP thymocytes are efficiently deleted. Furthermore, on C57BL/6 stroma, DP thymocytes are not selected to the CD4SP lineage (data not shown).

We also asked whether expression of Lck-GFP or CD3 ζ -GFP alters the ability of DP thymocytes to undergo positive and negative selection. After 72 hr in culture, examination of CD4/CD8 profiles of GFP-positive cells reveals that both positive and negative selection occur as efficiently on transduced thymocytes as on nontransduced cells (Figure 2B, lower panels). As previously described, the CD3 ζ -GFP fusion protein associates with ZAP70, is phosphorylated upon activation, and fails to alter the proliferative response to agonist when expressed in D10 T cells (Krummel et al., 2000). Likewise, Lck-GFP restores calcium flux to TCR crosslinking in JCAM1.6 cells and also fails to alter the proliferative response to agonist when expressed in D10 T cells (L.I.R., unpublished data). Therefore, expression of Lck-GFP and CD3 ζ -GFP in thymocytes does not detectably alter their ability to undergo proper signaling through the TCR.

Both Lck-GFP and CD3 ζ -GFP Are Accumulated at the Synapse during Negative Selection

To analyze synapse formation in reaggregate cultures, Lck-GFP and CD3 ζ -GFP expressing 5C.C7 DP thymocytes were reaggregated with B10.BR stroma with or without 1 μ M MCC(88-103). After 4 hr in culture, the time point at which the reagggregates had solidified sufficiently to be removed from the wells intact and at which surface molecules such as CD4 were recovered post-trypsinization, reagggregates were imaged. In reagggregates containing MCC(88-103) peptide, numerous Lck-GFP and CD3 ζ -GFP-expressing thymocytes formed conjugates with larger epithelial cells (Figures 3A and 3B). Thymocytes were scored positive for conjugate formation if they had flat interfaces in the GFP channel. These flat sides invariably colocalized with contact regions between thymocytes and epithelial cells in corresponding differential interference contrast (DIC) images, except where DIC images could not be resolved due to crowding of cells in the field. Ninety-eight percent of Lck-GFP conjugates and 95% of CD3 ζ -GFP conjugates displayed GFP accumulations of at least 1.3-fold at the contacting interface relative to noncontacting sides of the cells (average fold accumulation at the interface was 1.6 for Lck-GFP and 1.5 for CD3 ζ -GFP; Table 1). In DIC images, we frequently observed rosettes of thymocytes surrounding epithelial cells (Figure 3A, left panel), indicating that one epithelial cell could efficiently interact with multiple thymocytes. Thus, in the presence of an agonist peptide that mediates negative selection, thymic epithelial cells are able to efficiently form conjugates with 5C.C7 DP thymocytes. These conjugates demonstrate

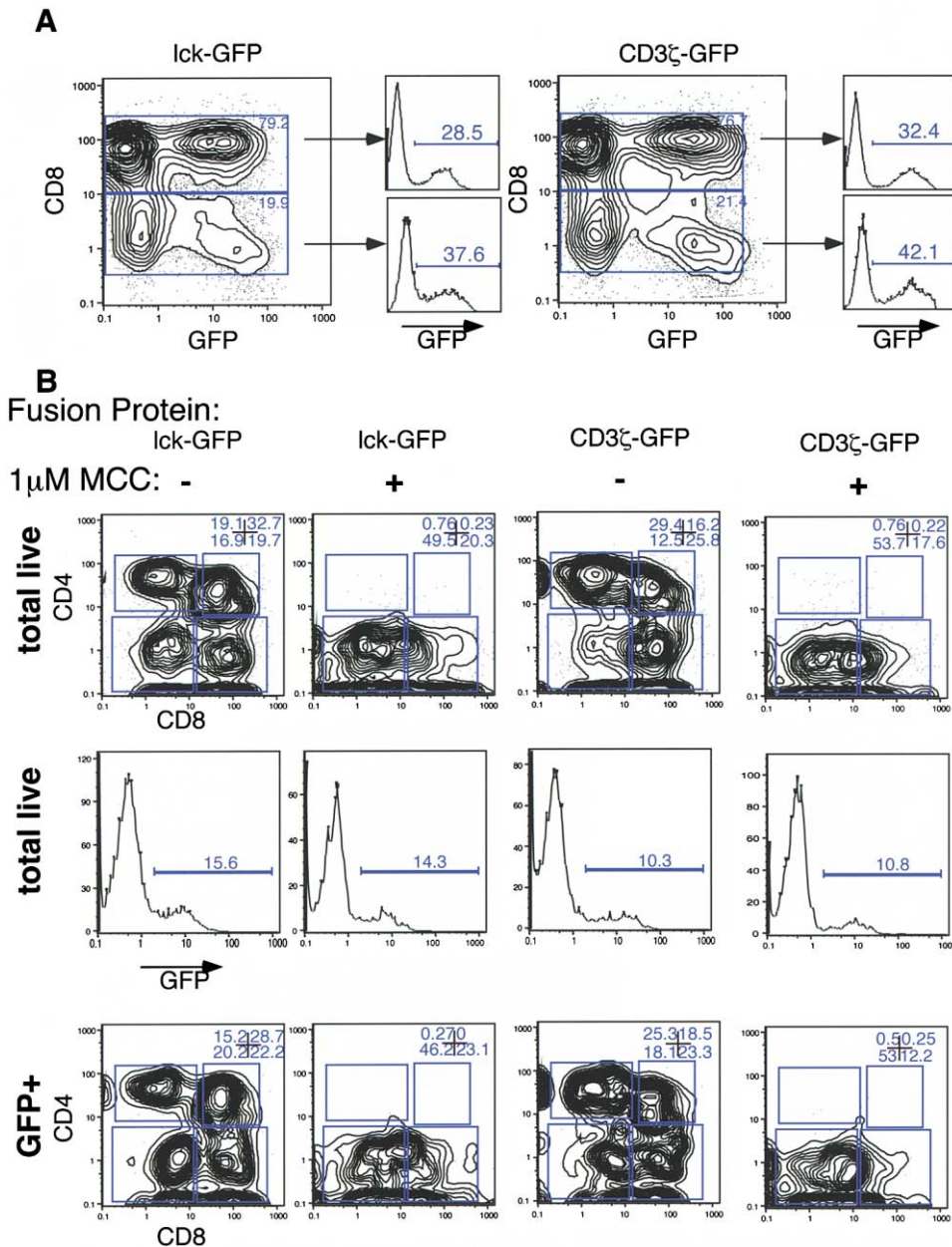


Figure 2. B10.Br Stroma Mediate Positive and Negative Selection of Lck-GFP and CD3 ζ -GFP-Expressing 5C.C7 TCR Transgenic Thymocytes (A) Six-day-old 5C.C7 C57BL/6 thymocytes were transduced with lck-GFP (left) and CD3 ζ -GFP (right) and stained for CD8 to delineate DP from DN cells. CD8⁺ DP and CD8⁻ DN cells were gated and analyzed for expression of GFP fusions. The percentage of GFP⁺ cells is shown above each histogram.

(B) Twenty-four hours after infection, thymocytes from an experiment comparable to (A) were enriched for DPs and reaggregated with 2-dGuo-treated B10.Br stroma \pm 1 μ M MCC(88-103) for 72 hr. Reaggregates were disrupted and stained for CD4 and CD8 expression. The top row shows profiles of total live cells, demonstrating that CD4SP cells are positively selected without peptide addition and DP cells are deleted upon agonist peptide addition. DN cells result from incomplete enrichment for DP thymocytes, while generation of CD8 SPs from DNs is a feature of TCR transgenic thymocyte development in organ culture (Kersh et al., 2000). The middle row shows the percentage of GFP⁺ cells remaining in the reaggregate after 72 hr. In the bottom row, GFP⁺ cells were gated and analyzed for CD4 and CD8 expression. The GFP⁺ thymocytes undergo positive selection to the CD4 lineage on B10.Br stroma and undergo negative selection of DP thymocytes upon addition of 1 μ M MCC.

immunological synapse formation with lck-GFP and CD3 ζ -GFP accumulated at the contact region (Figures 3A and 3B).

Since I-E^k molecules on B10.BR thymic stromal cells display exogenous MCC peptide as well as endogenous

peptides that might foster T cell interactions, we used a construct in which the I-E^k α chain was fused to an MCC peptide to show that MCC:I-E^k complexes drive synapse formation. As C57BL/6 mice lack a functional I-E α chain, expression of this construct in thymic stromal

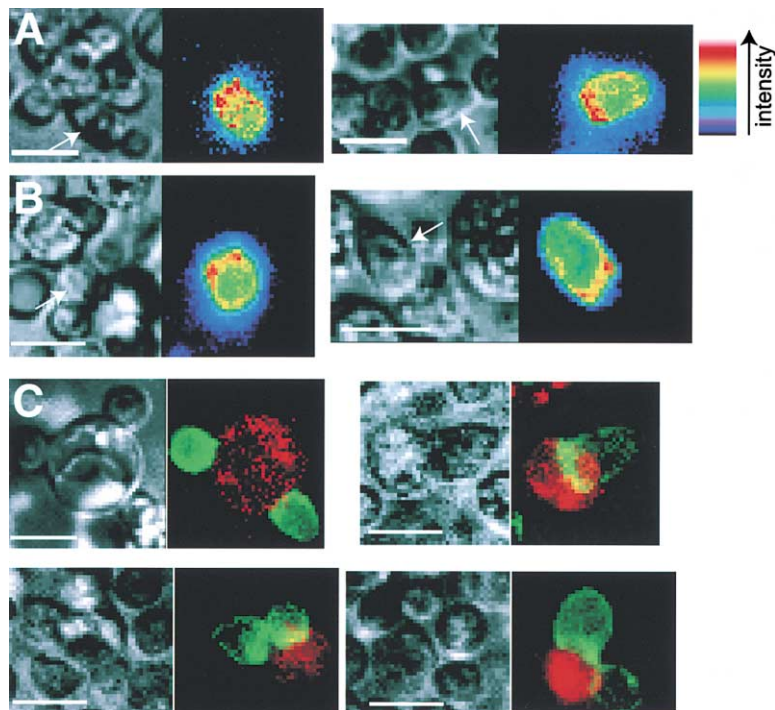


Figure 3. 2-dGuo-Treated I-E^k-Positive Stroma + MCC(88-103) Is Able to Mediate Conjugate Formation

(A and B) Transduced 5C.C7 DP thymocytes were reaggregated with 2-dGuo-treated B10.Br stroma + 1 μM MCC 4 hr prior to imaging. Each panel consists of a DIC image and a false-color GFP image of Ick-GFP (A) or CD3ζ-GFP (B). White arrows indicate the thymocyte shown in the GFP image. Note flat front and accumulation of GFP at the interfaces between thymocytes and stromal cells. A characteristic ring of thymocytes around a central epithelial cell can be seen in the left panel of (A).

(C) C57BL/6 stroma was transduced with MCC:I-E^α-CFP, cultured in 2-d-Guo for 1 week, and reaggregated with CD3ζ-GFP-transduced 5C.C7 DP thymocytes. Each panel consists of a DIC image and an overlay of CD3ζ-GFP (green) and MCC:I-E^α (red). Note that multiple small thymocytes frequently interact with a larger stromal cell as in the upper left panel. Scale bar, 10 μm. Supplemental Movie S1 (<http://www.immunity.com/cgi/content/full/16/4/595/DC1>) is a QuickTime movie showing a 3D rotation of cells shown in (C).

cells restores expression of I-E with MCC bound in its peptide binding groove. We also expressed a comparable construct lacking the MCC peptide. Both constructs encoded CFP at the carboxyl terminus of MHC.

C57BL/6 stromal cells transduced with either construct were maintained in 2-dGuo for 1 week prior to imaging. At this time, Ick-GFP- and CD3ζ-GFP-expressing 5C.C7 DP thymocytes were reaggregated with transduced stromal cells or with uninfected C57BL/6 stromal cells for 4 hr and then imaged. In reaggregates with uninfected C57BL/6 stromal cells, no conjugates were observed (data not shown), as would be expected since I-E is not expressed. In addition, no conjugates were observed on C57BL/6 stromal cells infected with the construct lacking MCC peptide. This situation mimics that of B10.Br stroma, which expresses I-E^k loaded with endogenous peptides, where we were able to see very few conjugates forming. However, conjugates were readily observed in reaggregates containing MCC:I-E^k-expressing stromal cells. Strikingly, for every conjugate observed in the GFP channel, there was a MCC:I-E^k-CFP expressing stromal cell to which the thymocyte was attached (Figure 3C). Frequently, we observed several

thymocytes interacting with one CFP-positive stromal cell (Figure 3C). Since the vast majority of thymocytes from 5C.C7 TCR transgenic mice express the transgenic TCR (Ho, 1995; Baldwin et al., 1999), we conclude that conjugates observed in our reaggregate systems are composed predominantly of 5C.C7 TCR transgenic DP thymocytes interacting with the negatively selecting ligand MCC:I-E^k on thymic epithelial cells.

Synapse Formation under Positively Selecting Conditions Is Inefficient

To ascertain whether synapses could be observed on thymocytes undergoing positive selection, transduced thymocytes were reaggregated with B10.Br stroma without peptide addition. As seen in Figure 2, these conditions generate a stromal environment that mediates efficient positive selection of 5C.C7 TCR transgenic thymocytes. However, we found very few conjugates, as scored by flat interfaces, under positively selecting conditions (Table 1). ≤4% of the GFP⁺ cells in several random fields demonstrated a conjugate phenotype after 4 hr of reagggregation with B10.Br stroma, while 40% demonstrated conjugate formation on B10.Br stroma + 1 μM MCC.

Table 1. Extent of Accumulation at the Interface during Positive/Negative Selection

	Lck-GFP + 1 μM MCC	Lck-GFP No peptide	CD3ζ-GFP + 1 μM MCC	CD3ζ-GFP No peptide
Number of Conjugates Scored	136	28	151	14
Average Accumulation	1.6	1.3	1.5	1.4
Minimum Accumulation	1.1	.9	1	.8
Maximum Accumulation	3.2	2	2	2.1
Percentage of Accumulations ≥ 1.3	98%	64%	95%	57%

All conjugates were scored for flat interfaces. Conjugates were then measured for the ratio of average GFP intensity at the interface over the side of the cell.

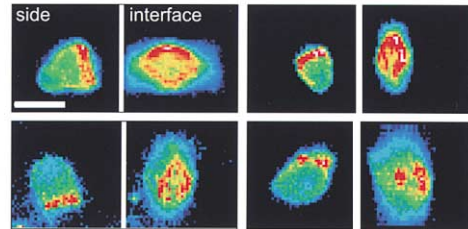
The extent of Ick-GFP and CD3 ζ -GFP accumulation at the interface on positively selecting conjugates was consistently slightly less than on negatively selecting conjugates (Table 1); only 57%–64% of positively selecting conjugates demonstrated an accumulation at the interface of ≥ 1.3 -fold, while nearly all conjugates under negatively selecting conditions had at least a 1.3-fold accumulation at the interface (Table 1). We were unable to observe any conjugates on B6 stroma.

Distinct Accumulation Patterns of Ick and CD3 ζ in Synapses of 5C.C7 Thymocytes and T Cell Blasts Undergoing Conjugate Formation

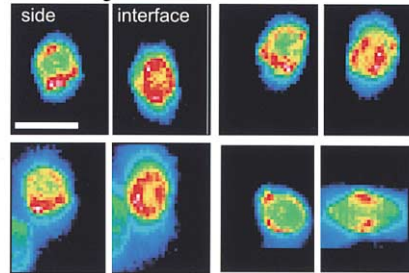
In order to examine the spatial organization of Ick-GFP and CD3 ζ -GFP at thymocyte:stromal cell interfaces under negatively selecting conditions, we collected stacks of GFP images at 1 μ m intervals and used these to reconstruct 3D images of conjugated thymocytes. The reconstructions were rotated to view the interacting surfaces of thymocytes and stromal cells (Figure 4). The thymocyte:APC interface was clearly delineated morphologically in the 3D GFP reconstructions as a flattened plane where the thymocytes were in contact with a stromal cell (see Supplemental Movies S2 and S3 at <http://www.immunity.com/cgi/content/full/16/4/595/DC1>). This allowed us to define regions within the interface such that accumulations that extended to the borders of the flattened interface but were excluded from the center were designated as peripheral accumulation patterns, while those in which the accumulated molecules were within the boundaries of the flat interface but not extending to those boundaries were designated as central accumulation patterns (for more detailed delineation of patterns, see Figure 5). Strikingly, the same predominant pattern of accumulation was seen for both Ick-GFP and CD3 ζ -GFP: both molecules were accumulated to a higher extent at the synapse periphery than at the center, resulting in a partial to complete ring of molecules around the center of the synapse (Figure 4 and Supplemental Movie S2 at the above URL). This pattern strongly resembles that seen for CD4 and LFA-1:ICAM in mature T cell synapses, but is unlike that reported for CD3 ζ -GFP or TCR accumulations in mature immunological synapses (Monks et al., 1998; Grakoui et al., 1999; Krummel et al., 2000). The excluded pattern of CD3 ζ -GFP accumulation was also observed on C57BL/6 stroma expressing MCC:I-E (see Supplemental Movie S1 at the above URL). Since many (at least 2000/cell; data not shown) of the I-E molecules on the MCC:I-E-expressing C57BL/6 stroma are loaded with the tethered MCC peptide, the local concentration of agonist peptide:MHC complexes on these cells is high relative to B10.BR stromal cells loaded with 1 μ M MCC. Nonetheless, we see excluded patterns of CD3 ζ -GFP in both situations, indicating that this pattern is not due to concentrations of MCC-peptide:I-E^k that are too low to recruit CD3 ζ to the center of the synapse.

To ensure that the observed excluded patterns of Ick-GFP and CD3 ζ -GFP were not an artifact of GFP-fusion mislocalization, we confirmed the localization patterns with antibody staining. Nontransduced 5C.C7 DP thymocytes were reagggregated with B10.BR stroma in the presence of 1 μ M MCC for 4 hr, fixed, and stained with

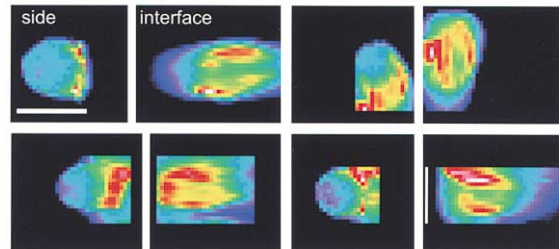
A Ick-GFP



B CD3 ζ -GFP



C anti-Ick



D anti-V β 3

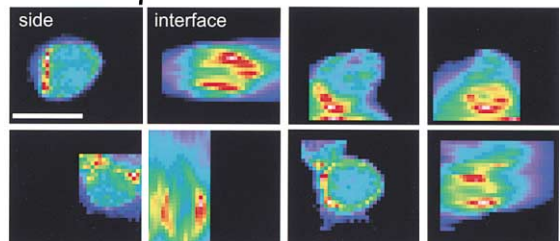


Figure 4. Ick and CD3 ζ Are Frequently Excluded from the Center of the Interface during Negative Selection

(A and B) Reagggregates of thymocytes expressing Ick-GFP (A) and CD3 ζ -GFP (B) were generated in the presence of 1 μ M MCC. 3D reconstructions of conjugated thymocytes were made; the left side of each panel shows a side view of the reconstructed cell, and the right side shows the interface en face.

(C and D) Reagggregates of 5C.C7 DP thymocytes and B10.BR stroma + 1 μ M MCC were generated, fixed, and stained with antibodies to Ick (C) or to V β 3 (D). 3D reconstructions of conjugated thymocytes were generated. Note that endogenous TCR and Ick are also distributed to the periphery of the interfaces. Scale bar, 10 μ m; color scale as for Figure 3.

Supplemental Movie S2 (<http://www.immunity.com/cgi/content/full/16/4/595/DC1>) is a QuickTime movie showing a 3D rotation of transduced thymocytes in (A) and (B).

antibodies to Ick and V β 3, the β chain variable segment of the 5C.C7 TCR. As seen in Figures 4C and 4D, both Ick and the TCR, respectively, were accumulated at the periphery of the interface between the thymocytes and

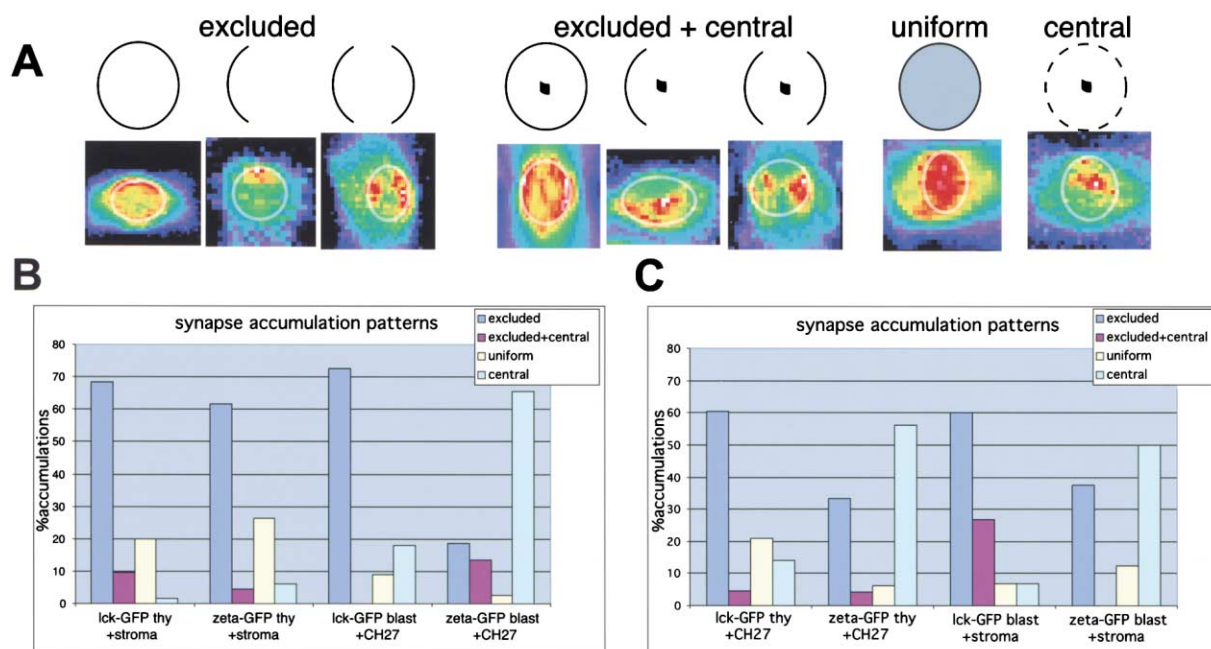


Figure 5. Lck-GFP and CD3 ζ -GFP Are Predominantly in Excluded Patterns in Thymocyte Conjugates, while CD3 ζ -GFP Is More Frequently Found in Central Accumulations in 5C.C7 Blast Conjugates

(A) Diagram and examples of accumulation patterns scored at immunological synapses. Circles outline the interface periphery as seen most clearly in Supplemental Movie S3 (<http://www.immunity.com/cgi/content/full/16/4/595/DC1>), which is a QuickTime movie of 3D rotations of different accumulation patterns.

(B) Reaggregates of thymocytes with 1 μ M peptide-loaded stromal cells formed for ≤ 4 hr, and reaggregates of T cell blasts with 1 μ M peptide-loaded CH27 cells formed for ≤ 2 hr. Accumulation patterns were scored and graphed for the four main accumulation patterns. Lck-GFP patterns are similar in thymocytes and blasts undergoing conjugate formation; however, CD3 ζ -GFP patterns differ markedly, as there are many more central patterns in blasts than in thymocytes. The ratios of peripheral:central GFP intensities for each accumulation pattern were consistent for all of the conjugates scored, indicating consistency in the designation of accumulation patterns (data not shown). Number of conjugates scored: 136 lck-GFP thymocytes, 151 CD3 ζ -GFP thymocytes, 44 lck-GFP blasts, and 81 CD3 ζ -GFP blasts.

(C) To assess the contribution of APC type to accumulation patterns, thymocyte:CH27 conjugates and blast:stromal cell conjugates were made with MCC preloaded APCs. CD3 ζ accumulation patterns are intermediate between those seen for thymocytes on stromal cells or blasts on CH27s, implicating a role for both APCs and T cell maturation stage in accumulation patterns. Color scale as for Figure 3.

MCC-pulsed stromal cells, confirming our results with the GFP-fusion proteins.

To quantify the frequency of different synapse patterns and to compare thymocytes directly with peripheral T cell blasts expressing the same TCR, we analyzed synapse patterns on numerous thymocyte:stromal cell conjugates and T cell blast:CH27 conjugates in the presence of MCC. We could identify four major types of synapse geometry with respect to both lck-GFP and CD3 ζ -GFP: excluded, excluded + central, uniform, and central, as illustrated in Figure 5A and Supplemental Movie S3 at <http://www.immunity.com/cgi/content/full/16/4/595/DC1>. Both lck-GFP- and CD3 ζ -GFP-expressing thymocytes primarily displayed excluded patterns (68.4% and 61.6%, respectively), while the majority of the remaining patterns for both were uniform accumulations (19.9% and 26.5%, respectively) (Figure 5B). Like lck-GFP-expressing thymocytes, the majority of conjugated lck-GFP-expressing blasts (73%) displayed excluded patterns of accumulation (Figure 5B). Thus, lck accumulation patterns seen during thymocyte negative selection and mature T cell activation appear similar. However, unlike the excluded accumulations of CD3 ζ -GFP-expressing thymocytes, accumulations of CD3 ζ -

GFP-expressing blasts at T:B interfaces were predominantly central (65.4%). A central pattern of TCR/CD3 accumulation following strong agonist signaling is a hallmark of other mature T cell systems, and corresponding central accumulations of class II MHC molecules on antigen-presenting cells have also been reported (Monks et al., 1998; Grakoui et al., 1999; Krummel et al., 2000). Thus, while CD3 ζ is recruited to synapses in both thymocytes undergoing negative selection and mature T cells undergoing activation, the patterns of accumulation are distinct.

Since different CD3 ζ -GFP accumulation patterns were observed between thymocyte:stromal cell conjugates and T cell blast:CH27 conjugates, it was unclear to what extent the developmental stage of the T cell versus the type of APC encountered contributed to the CD3 ζ -GFP accumulation patterns. To address this, we allowed thymocytes to form conjugates with MCC-pulsed CH27 cells and blasts to form conjugates with MCC-pulsed B10.BR thymic stroma and then assessed the lck-GFP and CD3 ζ -GFP accumulation patterns in the resultant synapses. As seen in Figures 5B and 5C, lck-GFP was predominantly in an excluded pattern regardless of the T cell developmental stage or type of APC used. How-

ever, in thymocyte:CH27 conjugates, there was an increase in the percentage of synapses that displayed a central accumulation for CD3 ζ -GFP in comparison to thymocyte:stromal cell conjugates (56% versus 6%, respectively). Conversely, T cell blast:stromal cell conjugates had a slight decrease in the percentage of central accumulations in comparison to blast:CH27 conjugates (50% versus 65.4%, respectively) (Figures 5B and 5C). However, the percentage of CD3 ζ -GFP central accumulations in thymocyte:CH27 conjugates, 56%, was still lower than that of blast:CH27 conjugates, 65%, and the percentage of CD3 ζ -GFP central accumulations in blast:stromal cell conjugates, 50%, was considerably higher than that of thymocyte:stromal cell conjugates, 6%. These findings indicate that while APCs play a role in determining the CD3 ζ -GFP accumulation pattern, the developmental stage of the T cell also contributes to the pattern of CD3 ζ -GFP accumulation. Furthermore, it should be noted that few conjugates were found between blasts and thymic stromal cells, and those that did form did not display the well-organized CD3 ζ -GFP excluded synapse found between thymocytes and stromal cells (see Figure 4), but rather displayed a fairly disorganized synapse with small accumulations of CD3 ζ -GFP (data not shown). Thus, stromal cells are particularly efficient at forming well-organized synapses with thymocytes with which they are physiologically in contact.

Synapses Form Rapidly during Negative Selection, Are Dynamic in Geometry, and Are Temporally Sustained

To observe synapse formation in real time in reaggregate cultures, we added MCC peptide 4 hr after reaggregate formation and no more than 10 min prior to imaging. Interestingly, we were able to see conjugates forming immediately. Given that only a tiny fraction of I-E^k complexes can be loaded with peptide under these conditions (C. Sumen, personal communication; Vacchino and McConnell, 2001; and data not shown), the rapid initiation of conjugate formation suggests that only a very small number of MCC:I-E^k complexes are able to trigger DP thymocytes to initiate signaling that results in negative selection.

Time-lapse movies of conjugate formation were acquired for 1 hr, with 3 min intervals between acquisitions. Both Ick-GFP and CD3 ζ -GFP accumulated within 3 min after conjugate initiation, identified as the time point at which a flat interface was visualized in the GFP image (see Supplemental Movie S4 at <http://www.immunity.com/cgi/content/full/16/4/595/DC1>). Therefore, recruitment of Ick and CD3 ζ to thymocyte:stromal cell interfaces during negative selection occurs as rapidly as recruitment to mature T cell:APC interfaces (Krummel et al., 2000). Accumulation patterns were dynamic for both Ick and CD3 ζ (Figure 6). While an excluded accumulation pattern was observed for the majority of time points for both molecules, boluses of Ick and CD3 ζ sometimes appeared in the center of the interface to generate uniform, excluded + central, and central accumulation patterns (Figure 6 and Supplemental Movie S4 at the above URL). Central accumulations appeared randomly throughout the life span of the conjugate and

were not sustained (data not shown). This indicates that the central accumulation patterns seen in a minority of thymocytes (Figure 5) are most likely not sustained either. In conjugates observed continuously for up to 1 hr, CD3 ζ accumulation was largely in an excluded pattern and in no case was there a stable central accumulation.

It has been suggested that negative selection is induced by strong but transient signals in thymocytes (reviewed in Hogquist, 2001). However, we observed conjugates in our time-lapse experiments that were stable for at least 1 hr. These data, in conjunction with the observation of stable Ick-GFP and CD3 ζ -GFP conjugates after 7 hr in reaggregate cultures containing 1 μ M MCC, indicate that thymocytes undergoing negative selection make stable, long-lived cell couples with thymic epithelial cells presenting negatively selecting peptide:MHC complexes. While it is possible that induction of apoptosis is very rapid, this long period of engagement is more reminiscent of synapses formed by mature T cells and may be necessary for a final commitment.

Discussion

By generating a defined negatively selecting environment for 5C.C7 TCR transgenic thymocytes, we have been able to image conjugate formation and recruitment of Ick and CD3 ζ to the immunological synapse at the outset of negative selection. While there has been some debate about the ability of thymic epithelial cells to mediate negative selection (Benoist and Mathis, 1989; Speiser et al., 1989), several groups have demonstrated that they are capable of mediating efficient negative selection with endogenous peptides (Bonomo and Matzinger, 1993; Volkman et al., 1997). In agreement with these results, we find that macrophage-depleted 2-dGuo-treated stromal cells, which are predominantly epithelial, can negatively select 5C.C7 TCR transgenic DP thymocytes in reaggregate cultures containing agonist peptide (Figure 2B). In addition, these stromal cells can also mediate positive selection (Figure 2B). Furthermore, retroviral-mediated introduction of GFP-tagged Ick and CD3 ζ into DP thymocytes does not alter their ability to undergo positive or negative selection (Figure 2B). Therefore, we have a flexible system with which to observe the dynamics of molecular movements in DP thymocytes as thymic selection is initiated.

It has been suggested that positive and negative selection might differ in the length of interactions between thymocytes and stromal cells (Hogquist, 2001). According to this "duration model," negatively selecting signals are strong and short lived, while positively selecting signals are weaker and long lived. We were able to observe the initiation of thymocyte:stromal cell conjugation in real time by adding MCC(88-103) to the reaggregate just prior to imaging. We observe conjugates that last for at least 1 hr (see Supplemental Movie S4 at <http://www.immunity.com/cgi/content/full/16/4/595/DC1>), and many conjugates are also present at least 7 hr after MCC addition, indicating that negatively selecting interactions are long lived. There are numerous reports that multiple or sustained interactions of the TCR with ligand are necessary for thymocytes to complete

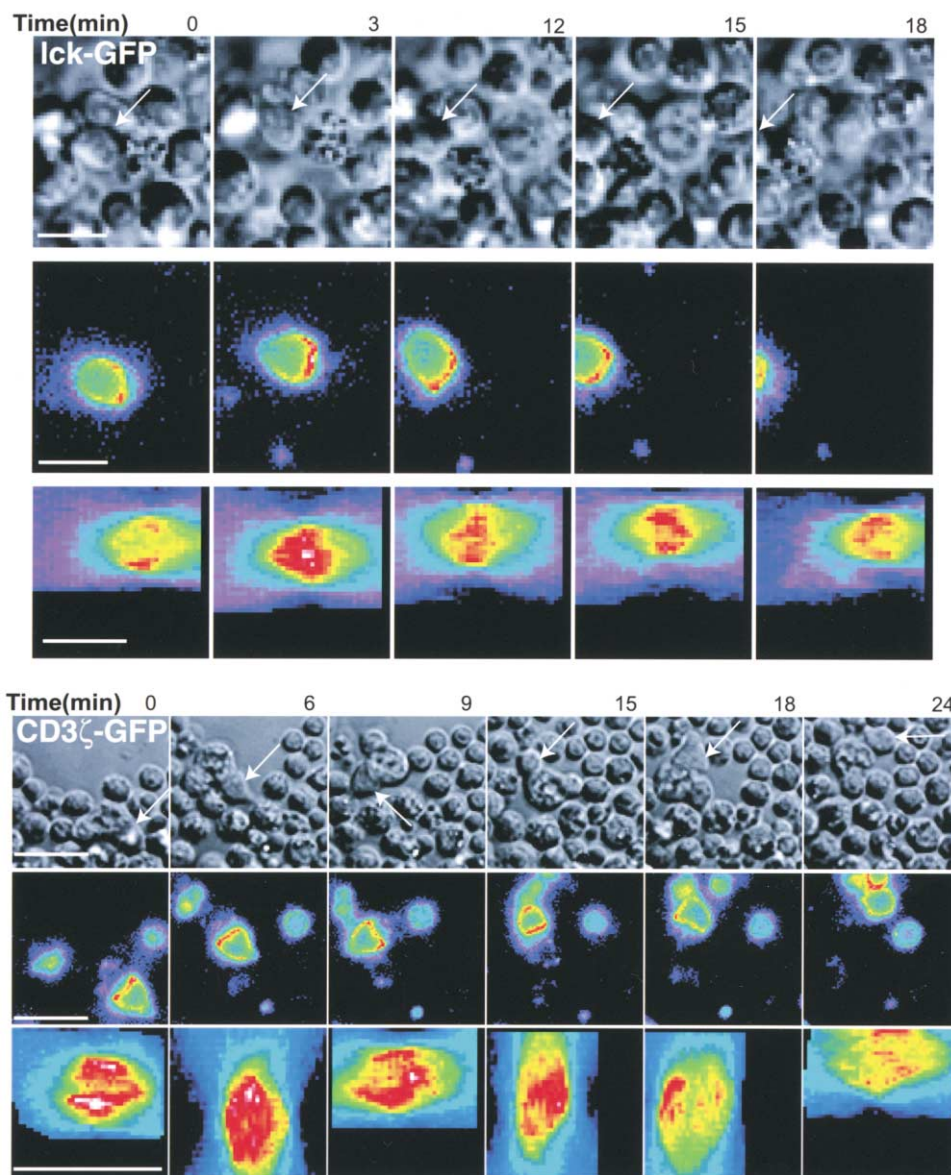


Figure 6. *lck*-GFP and CD3 ζ -GFP-Expressing Thymocytes Initiate Conjugate Formation with Negatively Selecting Stroma Rapidly, Demonstrate Dynamic Accumulation Patterns at the Interface, and Maintain Stable Interactions with Stromal Cells Over Time

5C.C7 DP thymocytes expressing *lck*-GFP (top) or CD3 ζ -GFP (bottom) were reaggregated with B10.Br stroma. MCC(88-103) peptide (1 μ M) was added within 10 min of imaging. Time-lapse images were acquired every 3 min for 1 hr. The top row consists of DIC images taken at the indicated time points. Time 0 corresponds to the first time a flat GFP interface was observed for that cell. The white arrow indicates the thymocyte undergoing conjugate formation. The middle row is a top view of a GFP image at one z plane in the middle of the cell; note the flat interfaces of conjugates. The bottom row is an en face view of the synapse. Note that both the *lck*-GFP and the CD3 ζ -GFP accumulation patterns are primarily excluded, but transient accumulations of both proteins do appear in the centers of the interfaces. Images are representative of 17 *lck*-GFP and 12 CD3 ζ -GFP movies. Scale bars, 10 μ m; color scale as for Figure 3. Supplemental Movie S4 consists of QuickTime movies of conjugates with 3 min between each frame. Upper left panel, DIC; upper right panel, top view of GFP image; and lower right panel, en face view of interface after 3D reconstruction. Time bar (min) is stamped in the lower left.

positive and negative selection (Kisielow and Mizsek, 1995; Wilkinson et al., 1995). Additionally, some studies argue that thymocytes undergo positive selection first and then proceed to negative selection (Lucas and Germain, 2000; Yasutomo et al., 2000). However, other studies indicate that negative selection can occur throughout thymocyte development without prior positive selection (Baldwin et al., 1999). Our studies concern the first 7 hr of thymocyte conjugate formation. Subsequent

TCR engagements or reorganization of the synapse may follow, and thus what we see may only reflect the initiation of negative selection. Interestingly, the rapid initiation of conjugate formation after MCC addition indicates that thymocytes are very sensitive to negatively selecting ligands, consistent with previous work showing that very low concentrations of antigen are sufficient to delete or tolerize thymocytes (Adelstein et al., 1991).

We also observed a small number of conjugates on

B10.BR stroma in the absence of peptide. As this stromal environment efficiently positively selects the 5C.C7 DP thymocytes (Figure 1), it is possible that the conjugates observed were undergoing positive selection, but we cannot rule out the possibility that these represent nontransgene-encoded TCRs reacting with endogenous peptide:I-E^k complexes. It is also possible that thymocytes are so sensitive to negatively selecting ligands that a small percentage transduce an inappropriate negative-selection signal due to rare encounters with a higher density of positively selecting ligands. Low efficiency conjugate formation is also seen during weak agonist stimulation of mature T cells (L.I.R., unpublished data; Krummel et al., 2000). Typically, these cells do not form flat interfaces with APCs. Thus, positive selection may resemble weak agonist stimulation in this system, and the molecular clusters of molecules that initiate TCR signaling may be so small as to be below the level of detection. Alternatively, if positively selecting peptides are expressed at low levels in the reagggregates, thymocytes may undergo positive selection in a temporally unsynchronized manner and thus may be difficult to detect.

The actual functions of T cell synapses are unknown. Recently, it has been suggested that the synapse does not play a major role in T cell activation, as phosphorylation and calcium elevation precede the stable central TCR cluster associated with a mature immunological synapse. In this view, synapse formation may be necessary for effector functions in mature T cells (Davis and van der Merwe, 2001; Lee et al., 2002). Since thymocytes lack effector functions, they may not normally progress to a central CD3 ζ accumulation. However, at least 1–2 hr of signaling through the TCR is necessary for a mature T cell to commit to activation (Kartunen and Shastri, 1991; Lee et al., 2002), in contrast to the much shorter time needed for synapse formation. Thus, some aspects of synapse formation are likely to be important to signaling, and the inability of CD3 ζ (and TCR) to form a stable, central accumulation in thymocytes undergoing negative selection (Figure 3) may lead to apoptosis rather than activation.

Could either quantitative or qualitative differences in CD3 ζ recruitment contribute to these different cell fates? Recruitment of Ick and CD3 ζ to thymocyte immunological synapses occurs to the same extent and with similar kinetics as recruitment to mature T cell synapses (Figure 6 and data not shown), and thus quantitative differences cannot explain the alternate cell fates. What we do see, however, are qualitative differences in the CD3 ζ accumulation patterns between thymocytes and mature T cells. In particular, thymocytes fail to form stable central accumulations over 1 hr of continuous observation, and they do not progress to this morphology as late as 7 hr after peptide addition (Figure 5). We conclude that stable central CD3 ζ accumulations do not occur in thymocytes initiating negative selection. This is in contrast to the recent work of Shaw and colleagues which shows that naive T cells first display a peripheral TCR accumulation followed by later (~30 min) central patterns (Lee et al., 2002). It is interesting that thymocytes in our system are similar in the early stages of activation to naive T cells, but not the later. Therefore, TCR and associated CD3 molecules may interact to a different extent with

molecules in thymocyte synapses during negative selection than in mature T cell synapses during activation. Perhaps physical separation of TCR/CD3 from molecules in the center of the synapse during negative selection results in different patterns or durations of ITAM phosphorylation (Love and Shores, 2000). Alternatively, physical separation of molecules into peripheral versus central regions of the synapse may allow for different molecular complexes to form in thymocytes undergoing negative selection than in mature T cells undergoing activation.

How might these different synapse patterns come about? One possibility is that there is a fundamental difference between thymic and peripheral APCs. B7:CD28 interactions facilitate formation of stable accumulations of signaling molecules at the center of mature T cell synapses, and blocking such costimulatory interactions impairs synapse formation (Wulfiging and Davis, 1998; Krummel et al., 2000; Wulfiging et al., 2002). However, thymic epithelial cells do not express B7 molecules, and B7:CD28 interactions have been shown to be unnecessary for negative selection (Jones et al., 1993). Thus, altered costimulation might be the cause of the differences seen here. Indeed, we find that there are more central CD3 ζ accumulations in thymocyte:CH27 conjugates than in thymocyte:stromal cell conjugates, indicating that APC type can influence synapse morphology. Nonetheless, thymic stromal cells are unable to induce strong conjugate formation with T cell blasts, and synapses that do form are generally disorganized. In contrast, thymocytes form highly organized synapses efficiently with stromal cells, displaying peripheral Ick and CD3 ζ accumulations (Figure 5). Therefore, both APC type and the T cell developmental stage contribute to synapse morphology. As stromal cells are a heterogeneous population, it remains possible that thymocytes and blasts preferentially interact with distinct stromal cell types, contributing to observed differences in CD3 ζ accumulation. It is also possible that cytoskeletal reorganization may occur differently in thymocytes and mature T cells, forcing signaling molecules into different compartments within the synapse. Indeed, cytoskeletal reorganization has already been shown to contribute to mature synapse formation (Wulfiging and Davis, 1998; Krummel et al., 2000; Davis and Cooper, 2000). Finally, lipid raft domains may be differently organized in thymocytes and mature cells such that constrictions are placed upon the diffusion of proteins in the plasma membrane relative to one another (Ebert et al., 2000; Gomez-Mouton et al., 2001).

In summary, we have established a reaggregate system together with retroviral transduction with which to image thymocyte-stromal cell interactions during thymic development. This system holds great promise in elucidating the dynamics of cellular interactions and the molecular movements that underlie selection and lineage determination events. Here we have studied the recruitment of Ick and CD3 ζ to the synapses that form between stromal cells and thymocytes undergoing negative selection. We find that the phenotype of CD3 ζ accumulation is distinct from that observed in mature T cell synapses, indicating significant cellular differences in the response of these cells upon encountering their ligands.

Experimental Procedures

GFP Fusion Retroviral Constructs and Propagation of Retroviruses

cDNAs for CD3 ζ (Krummel et al., 2000) and Ick (L.I.R., unpublished data) were cloned into EGFP-N1 (Clontech) to generate in-frame EGFP fusion protein constructs. To generate retroviral vectors, the cDNAs for Ick-GFP and CD3 ζ -GFP were subcloned into the retroviral vector pIB. pIB is derived from the MFG series of retroviral vectors, has full-length Moloney LTRs, an extended ψ sequence, and an IRES upstream of a blasticidin-s deaminase gene (a gift from Cenk Sumen). cDNAs for the I-E $^{\alpha}$ chain with and without tethered MCC were subcloned into pIB. The MCC peptide corresponds to residues (91–103) of MCC and is attached via a 32-amino-acid linker of (Gly-Gly-Gly-Ser) $_n$ to the N terminus of the I-E $^{\alpha}$ chain.

To create retroviruses, pIB retroviral vectors were transfected into Phoenix-ecotropic producer cells (a gift of Dr. Garry Nolan). Transfected Phoenix-E lines were stably selected and maintained in 10 μ g/ml Blasticidin (Invitrogen). The producer lines were monitored by FACS for GFP or CFP expression.

To collect virus for infection of thymocytes and stromal cells, Phoenix-E Ick-GFP and Phoenix-E CD3 ζ -GFP were grown at 37°C in 7% CO $_2$ to 60%–70% confluency on 10 cm tissue culture dishes in DMEM containing 10% FBS, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine and 10 μ g/ml Blasticidin. To collect retroviral supernatant, the media was replaced with RPMI containing 10% FBS, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, and 50 μ M β -mercaptoethanol, and plates were transferred to 32°C in 7% CO $_2$. After 48 hr, the media was removed, filtered through .45 μ m syringe filters (UNIFLO, Schleicher & Schuell) to remove any cells, and spun overnight at 4°C at 6000 \times g (adapted from Travers et al., 2001). Supernatant was aspirated off the viral pellet, the pellet was resuspended in 1/10 volume RPMI, passed several times through a 20G needle, and refiltered through .45 μ m syringe filters.

Preparation and Infection of Thymocytes and Blasts

5C.C7 TCR transgenic mice were maintained on C57BL/6 and B10.Br backgrounds. Offspring (6- to 10-days-old) of C57BL/6 mice heterozygous for the TCR transgene were sacrificed on the day of infection, and thymuses were removed. Thymocytes from mice determined to be transgene positive (by staining with anti-V β 3 antibody, KJ25; Pharmingen) were transduced with Ick-GFP or CD3 ζ -GFP retroviruses according to a modified protocol from Travers et al. (2001). Briefly, 3 \times 10 6 thymocytes were resuspended in 1 ml of concentrated retroviral supernatant plus 5 μ g/ml polybrene (Sigma) and 10 ng/ml recombinant mouse IL-7 (R&D Systems) in a 24-well plate. The plate was then spun at 32°C at 2500 rpm in a Beckman table-top centrifuge for 1 hr and placed overnight in a 37°C incubator.

To prepare blasts, lymph nodes were dissected from 4- to 6-week-old 5C.C7 TCR transgenic mice on a B10.Br background, and single-cell suspensions were made. Lymphocytes (3 \times 10 6) were placed in each well of a 24-well dish with 1 μ M MCC(88-103) and incubated at 37°C. Twenty-four hours after activation, the blasts were resuspended in concentrated viral supernatant + 5 μ g/ml polybrene + 1% IL-2. The plate was spun as above, and 24 hr later the infected cells were removed into fresh RPMI. Forty-eight to seventy-two hours after the media change, the blasts were imaged for conjugate formation after mixing with CH27 cells or with thymic stromal cells loaded \geq 1 hr with 1 μ M MCC.

Preparation and Infection of Stromal Cells

To prepare B10.Br stromal cells, thymic lobes from 14- to 15-day-old B10.Br embryos were placed in organ culture in complete RPMI containing .36 mg/ml 2-deoxyguanosine (Jenkinson et al., 1992). After 5–6 days in organ culture, lobes were disrupted with .25% trypsin in .02% EDTA (Sigma) for 15 min at 37°C. To deplete macrophages, anti-CD45 (30.11, Pharmingen)-coated Streptavidin beads (Dyna) were mixed with stromal cells and spun twice at 1000 rpm for 3 min in a table-top centrifuge. The cells were placed on the magnet 2 \times for several minutes, and the unbound cells were collected into a new eppendorf tube.

To infect C57BL/6 stromal cells, thymic lobes from day 15–16

C57BL/6 embryos were disrupted with trypsin EDTA for 15 min at 37°C and depleted of CD45-positive cells. The remaining stromal cells were spin infected with MCC:I-E $^{\alpha}$ -CFP or I-E $^{\alpha}$ -CFP retroviruses for 1 hr, spun to form reagggregates in 96-well v-bottom plates, incubated overnight at 37°C under enriched oxygen conditions (60% O $_2$), and then transferred to organ culture in 2-deoxyguanosine. Lobes were maintained in organ culture for 1 week prior to use in reagggregates.

Generation of Reagggregates and Conjugates for Imaging

After 24 hr infection with virus, thymocytes were enriched for DP cells by positively selecting on anti-CD8-coated streptavidin Dynal beads (clone 53.67, Pharmingen). The cells were treated with trypsin EDTA at 37°C for 15 min to release the beads. To generate reagggregates with B10.Br stroma, 2.5 \times 10 5 stromal cells and 1.5 \times 10 5 infected DP 5C.C7 thymocytes were placed in 96-well v-bottom plates in complete RPMI +/- 1 μ M MCC(88-103). The plates were spun at 1000 rpm for 10 min and 2000 rpm for 10 min, then sealed in a humidified box that was filled with 60% O $_2$ and placed at 37°C for 4 hr until they were imaged. To generate reagggregates with transduced B6 stroma, 1.5 \times 10 5 stromal cells and 1 \times 10 5 infected DP 5C.C7 thymocytes were reagggregated as described above.

To examine thymocyte:CH27 conjugates, transduced 5C.C7 DP thymocytes were mixed 2:1 with CH27 cells that had been pulsed for \geq 1 hr with 1 μ M MCC. The cells were gently centrifuged to encourage conjugate formation, incubated for 10 min at 37°C, and then placed in chamber slides for immediate imaging.

Antibody Staining

Reagggregates of freshly isolated 5C.C7 DP thymocytes and B10.Br stromal cells were generated as described for transduced thymocytes, incubated at 37°C for 4 hr, and fixed in 3.5% paraformaldehyde. To stain for endogenous V β 3, KJ25PE (BD Pharmingen) was incubated with the reaggregate for 1 hr. To stain for endogenous Ick, reagggregates were permeabilized in .3% saponin for 1 hr prior to a 1 hr incubation with a monoclonal anti-Ick antibody (3A5; Santa Cruz Biotechnology). This was followed by a 1 hr incubation with Cy-3-conjugated anti-mouse Ig, Fc γ fragment-specific antibody (Jackson ImmunoResearch). Reagggregates were then imaged.

Imaging

Reagggregates were carefully pipetted from v-bottom wells onto the glass slide of a Biopetechs FCS2 chamber. The reaggregate was sandwiched between a coverslip, and the slide was separated by a .25 mm gasket. Using mechanical pressure, the reaggregate structure was slightly disrupted to allow cells to spill out for facilitated imaging.

Imaging was carried out using a Zeiss Axiovert-100TV microscope and a Plan Neo-Fluar 40 \times /1.3 objective. The microscope was fitted with a high-speed piezo electric z-motor (Physik Instruments), dual excitation and emission filter wheels (Sutter Instruments), and a Princeton Instruments Interline camera (Roper Scientific). A high-intensity Xenon light source (Sutter Instruments) was utilized, and data were streamed from the camera to the computer to eliminate excessive bleaching. For time-lapse experiments, images were taken every 3 min for 1 hr. At each time point, we collected a DIC image followed by a 20 μ m z stack of GFP images separated by 1 μ m. Microscope control, data acquisition, and image analysis were performed in Metamorph (Universal Imaging). Conjugates in which the plane of the interface was not orthogonal to the z stacks were disregarded during analysis.

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