

to phospho-p44/p42 MAPK (Thr<sup>202</sup>/Tyr<sup>204</sup>) (1:5000), and pAb to phosphoAkt (Ser<sup>473</sup>) (1:1000) (Cell Signaling Technology, Beverly, MA). Gel loading of total TrkA and MAPK was determined by immunoblotting with pAb to TrkA (C-14) (1:2000) and pAb to p44 MAPK (C-16) (1:2000) (Santa Cruz Biotechnology, Santa Cruz, CA), which reacts with both p44 and p42 MAPK. Immunoreactivity was detected by enhanced chemiluminescence (SuperSignal West Dura Substrate, Pierce, Rockford, IL). Blots were stripped with Restore Western Blot Stripping Buffer (Pierce) according to the manufacturer's directions.

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23. Neuronal survival was assessed by Hoechst staining of DNA. Cultures were fixed with 4% paraformaldehyde at room temperature for 20 min and incubated in Hoechst 33258 dye (50 mM in PBS; Molecular Probes) for 30 min. Confocal images were obtained with a Zeiss LSM 510 laser scanning microscope (Carl Zeiss, Jena, Germany). Stained nuclei were quantified

using a Nikon Eclipse TE300 inverted fluorescence microscope (Nikon, Tokyo, Japan).

24. Mouse NGF (2.5S, grade II; Alomone Labs, Jerusalem, Israel) was commercially conjugated to <sup>125</sup>I by ICN Radiochemicals (Irvine, CA) using the lactoperoxidase/H<sub>2</sub>O<sub>2</sub> method without carrier protein. Unconjugated NGF and <sup>125</sup>I were removed by HPLC, yielding greater than 97% purity of <sup>125</sup>I-NGF with a specific activity at the time of the experiments of 38.2 mCi/mg. Transported <sup>125</sup>I-NGF was measured by  $\gamma$ -counting the medium bathing the cell bodies/proximal axons (7, 15). Control cultures for nonspecific transport were given medium containing <sup>125</sup>I-NGF plus 100 $\times$  excess of unlabeled NGF; and mean nonspecific transport, which was less than 5% of transport obtained with free <sup>125</sup>I-NGF (50 ng/ml), was subtracted from mean transport of each group. The <sup>125</sup>I-NGF and <sup>125</sup>I-NGF beads within an experiment were from the same preparation.

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## T Cell Receptor Signaling Precedes Immunological Synapse Formation

Kyeong-Hee Lee,<sup>1</sup> Amy D. Holdorf,<sup>1</sup> Michael L. Dustin,<sup>2</sup> Andrew C. Chan,<sup>3</sup> Paul M. Allen,<sup>1</sup> Andrey S. Shaw<sup>1\*</sup>

The area of contact between a T cell and an antigen-presenting cell (APC) is known as the immunological synapse. Although its exact function is unknown, one model suggests that it allows for T cell receptor (TCR) clustering and for sustained signaling in T cells for many hours. Here we demonstrate that TCR-mediated tyrosine kinase signaling in naïve T cells occurred primarily at the periphery of the synapse and was largely abated before mature immunological synapses had formed. These data suggest that many hours of TCR signaling are not required for T cell activation. These observations challenge current ideas about the role of immunological synapses in T cell activation.

The biochemical pathways that are stimulated by TCR engagement are well characterized, but little is known about how T cell interactions with the APC actually initiate signaling by the TCR. The immunological synapse hypothesis proposes that membrane protein reorganization at the T cell–APC contact surface serves to generate a structure, the immunological synapse, which facilitates TCR signaling by concentrating both TCRs and antigenic major histocompatibility complex (MHC)–peptide complexes as well as lipid rafts in the center of the contact [reviewed in (1, 2)]. The stability of this structure can

explain how TCR engagement can be sustained for long periods of time (3).

The immunological synapse was first described as having two discrete zones by using high-resolution immunofluorescence imaging of T cell–APC conjugates (4). The central zone, the c-SMAC, contains the TCR and surface accessory molecules such as CD4, CD2, and CD28 (3). Surrounding the central zone is a second zone, the p-SMAC, which is enriched for the integrin, LFA-1. Real-time imaging with T cells plated on planar membranes shows that synapse formation occurs with TCRs first engaged by MHC peptide in the periphery of the synapse followed by recruitment of such complexes to the center of the synapse. To date, however, the initial engagement of the TCR in the periphery of the contact has not been visualized in immunological synapses formed with APCs (2). We wished, therefore, to determine where TCR–MHC engagement first occurs during authentic synapse formation and to compare it with known T cell signal transduction events.

Freshly isolated naïve T cells were im-

aged to most closely mimic conditions in vivo. We also reasoned that the higher threshold for activation of naïve T cells might result in slower immunological synapse formation. Naïve T cells were purified from AND TCR transgenic mice that recognize a moth cytochrome c peptide (residues 88 to 103) presented by I-E<sup>k</sup> (5). Freshly isolated T and B cell–depleted splenocytes were used as APCs. Conjugate formation was initiated by centrifuging together T cells and APCs, which had been preincubated with antigenic peptide (6). After various times, cells were fixed and then immunostained for TCR and LFA-1 (6). At the earliest time points (2 through 15 min), we noted that the majority of LFA-1 molecules were recruited to the center of the contact area, whereas TCRs were concentrated at the periphery of the synapse (Fig. 1A). Between 15 and 30 min, this pattern reversed, with TCRs now visible in the center of the contact surrounded by an external ring of LFA-1 (Fig. 1A). By 60 min, TCRs in the synapse were not detectable even though LFA-1 was still concentrated in the synapse. Computer reconstructions of the synapse (from serial confocal images along the z axis) confirmed the peripheral localization of the TCR at early time points (15 min), referred to as an immature immunological synapse, and the central localization of the TCR at later time points (30 min), referred to as a mature immunological synapse (Fig. 1B). Immature synapses were also detected with T cell blasts, but synapse maturation occurred much more rapidly (within 1 to 3 min) and the magnitude of TCR down-regulation was much less (7).

To relate the events in the formation of the immature and mature immunological synapse to TCR-mediated signaling, we used phospho-specific antibodies that recognize the activated forms of two key TCR-regulated cytoplasmic tyrosine kinases, Lck and ZAP-70.

<sup>1</sup>Department of Pathology and Immunology, Washington University School of Medicine, 660 South Euclid, Box 8118, Saint Louis, MO 63110, USA. <sup>2</sup>Program in Molecular Pathogenesis, Skirball Institute of Molecular Medicine, New York University, 540 First Avenue, New York, NY 10016, USA. <sup>3</sup>Genentech, Department of Immunology, 530 Forbes Boulevard, One DNA Way, South San Francisco, CA 94080, USA.

\*To whom correspondence should be addressed. E-mail: shaw@pathbox.wustl.edu

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Lck is the first tyrosine kinase activated during TCR signal transduction and is responsible for phosphorylating key tyrosine residues in the TCR (8). Its autophosphorylation on tyrosine residue 394 correlates with its activation (9). Cell conjugates were fixed and

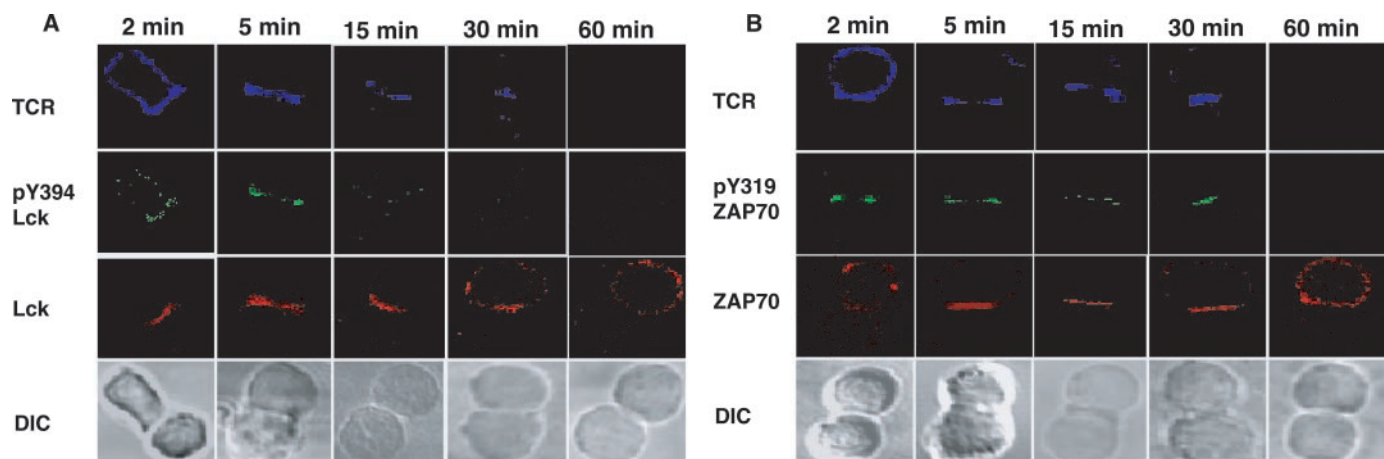
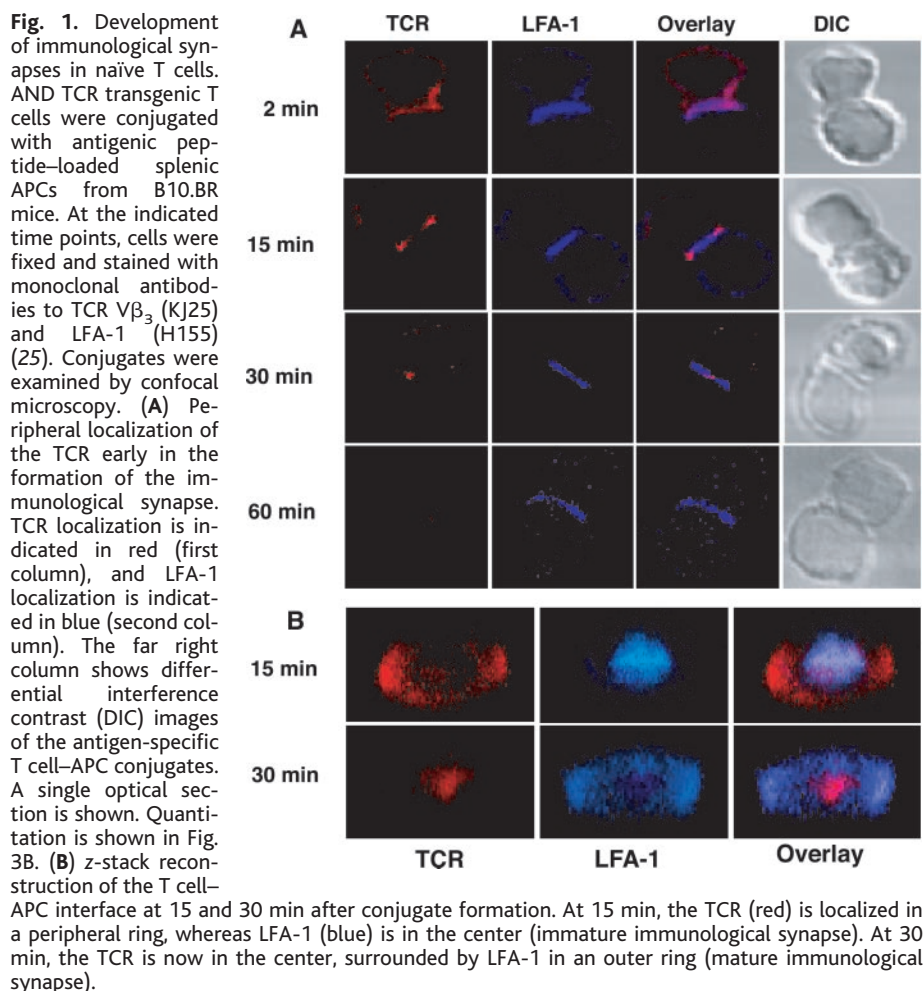
stained with antibodies to the TCR, active Lck ( $\alpha$ -pY394 antibody), and total Lck and imaged at various time points after conjugation (6). At early time points (Fig. 2A, 2 min), Lck was recruited to the synapse consistent with earlier reports (4). Over the next

15 to 30 min, Lck concentrated at the center of the contact. Between 30 and 60 min after conjugate formation, Lck dispersed from the contact area and was evenly distributed in the T cell plasma membrane.

Active Lck had a different pattern of staining. At early time points (2 to 15 min), active Lck colocalized with the external ring of TCR in immature synapses. The intensity of staining peaked between 5 and 7 min after conjugate formation and was undetectable 15 min after conjugate formation, well before the mature synapse had formed. No active Lck could be detected in the mature synapse, even though Lck is concentrated in the center. Taken together, these results demonstrate that Lck is recruited early to the synapse and is present in both immature and mature synapses. However, active Lck is only detected in immature contact at the periphery of the cell contact (Fig. 2A).

Lck phosphorylation of the ITAM sequence results in ZAP-70 recruitment and activation at the plasma membrane (8, 10, 11). T cell-APC conjugates were formed, fixed, and then stained with antibodies to TCR, active ZAP-70, and total ZAP-70. In comparison with Lck, recruitment of ZAP-70 to the synapse was delayed, not occurring detectably until at least 5 to 7 min after conjugate formation (Fig. 2B). Like Lck, ZAP-70 persisted at the center of the synapse for up to 30 min after conjugate formation and had dispersed into the T cell cytoplasm by 60 min.

Activation of ZAP-70 results in its phosphorylation on tyrosine-319 by Lck (11). Using a phospho-319 antisera, we first detected active ZAP-70 at the periphery of immature synapses and then weakly in the center of mature synapses (Fig. 2B). At 60 min, no active ZAP-70 was detected. Similar results with antibodies to phosphotyrosine con-



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firmed that tyrosine kinase signaling initiates at the periphery of immature synapses and decreases in intensity as the synapse matures (Fig. 3, A and B). Phosphotyrosine immunoblotting of zeta immunoprecipitations confirmed that tyrosine phosphorylation peaks at about 10 min (12). The initiation of TCR-mediated tyrosine kinase signaling, therefore, appears to occur early and largely precedes the formation of the mature immunological synapse. This is also consistent with previous biochemical studies demonstrating the kinetics of tyrosine kinase signaling (13).

Although TCR down-regulation from the surface during antigen engagement is well known (14), the magnitude of TCR down-regulation seen here was surprising given previous data suggesting that sustained TCR engagement, up to 20 hours in length, is required for activation of naïve T cells (15). Previous studies used [<sup>3</sup>H]thymidine incorporation to measure the commitment of T cells to proliferate. Because T cells undergo several rounds of cell division after activation, [<sup>3</sup>H]thymidine incorporation might overestimate the time required for cell commitment. Therefore, we evaluated this issue using a more sensitive method to measure cell cycle

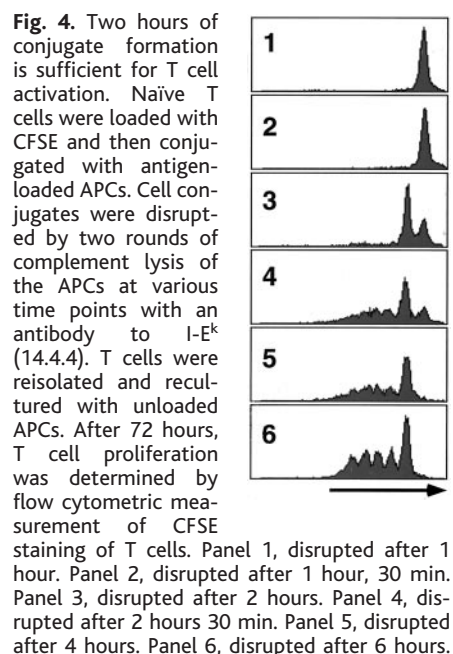
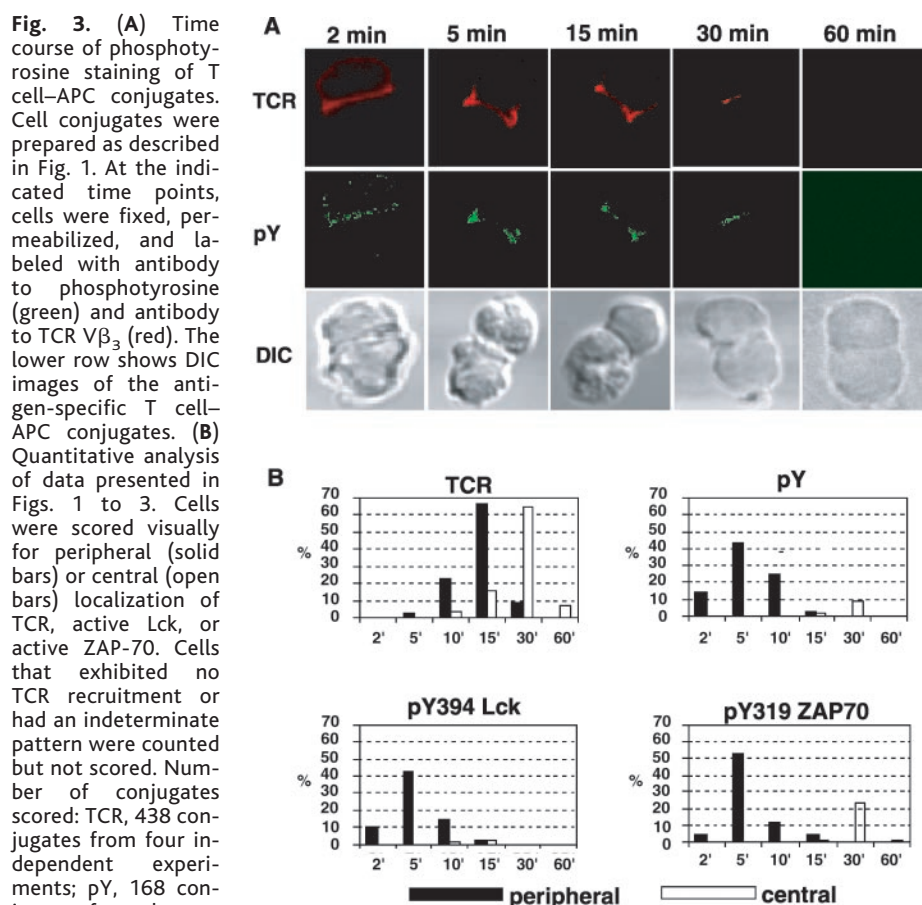
progression, carboxyfluorescein diacetate succinimidyl ester (CFSE) staining (16). This allowed us to focus only on the time of contact required to achieve the first cell division. T cells were loaded with CFSE before conjugate formation with APCs (17). After various times, APCs were removed by using two rounds of complement-mediated lysis with antibodies to MHC class II (12). T cells were placed back in culture and analyzed for CFSE staining at 24, 48, and 72 hours.

No change in CFSE staining was detected in T cells incubated with APCs for less than 2 hours, indicating that the cells had not divided (Fig. 4). After 2 hours, however, the decreased intensity of CFSE staining indicated that most of the T cells had divided. To confirm that a membrane structure did not persist on the T cell membrane after APC removal, we treated T cells with the cholesterol-depleting drug, methyl- $\beta$ -cyclodextrin (MCD), which disrupts lipid rafts. Although MCD treatment before APC engagement inhibited T cell proliferation, MCD treatment after 2 hours had no effect (12). Thus, the minimal duration of naïve T cell–APC interaction required for cell division is about 2 hours. The 2-hour requirement is consistent

with recent studies of naïve CD8<sup>+</sup> cells (18) and earlier studies of the length of engagement required for interleukin-2 release from T cells (19). One difference is our finding that 2 hours was only sufficient for a single cell division in naïve CD4<sup>+</sup> cells; in the study of naïve CD8<sup>+</sup> cells, 2 hours was sufficient for multiple cell divisions (18).

Here, we show that T cell engagement and activation begin before immunological synapses are fully formed. Phospho-specific antibodies to Lck and ZAP-70 demonstrated that the strongest signaling occurs before the mature synapse has formed. Although, Lck, ZAP-70, and some tyrosine-phosphorylated proteins were detectable at the center of the synapse, the magnitude of signaling was weak. Moreover, the rapid and profound loss of TCRs from the synapse by 30 min is consistent with the idea that TCR signaling peaks before the mature synapse has formed. Thus, the central clustering of the TCR generated by the immunological synapse formation does not function to initiate or sustain TCR signaling. Although only 2 hours of T cell–APC contact was required for T cell commitment, there is still a gap between our inability to detect tyrosine phosphorylation after 30 min and T cell commitment at 2 hours. Although it is certainly possible that residual tyrosine kinase signaling occurs below our level of detection, it is also possible that other signaling systems, such as those involving calcium or serine/threonine phosphorylation, may be critical during this period.

What then is the function of the immunological synapse? Davis and van der Merwe have argued that it is not involved in signal transduction but rather it is required for po-



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larized secretion (20). We favor the idea that the immunological synapse is involved in TCR down-regulation and endocytosis. TCR endocytosis might be required for signaling as proposed for the epidermal growth factor (EGF) receptor (21) or could function to attenuate TCR signaling responses (22). Finally, it is possible that the immunological synapse provides a surface allowing the activation and engagement of receptors other than the TCR. For example, cytokine signaling is potentiated in the context of focal adhesions (23). Secondary signaling pathways that are up-regulated after TCR triggering, such as ICOS and CTLA-4, might be facilitated by synapse formation (24). Clearly, a better understanding of the structure and function of the immunological synapse will lead to insights into the T cell activation process.

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