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Differences in Signaling Molecule Organization between Naive and Memory CD4$^+$ T Lymphocytes

Andrew R. O. Watson* and William T. Lee$^2$$^*$$^†$

The immunological synapse is a highly organized complex formed at the junction between Ag-specific T cells and APCs as a prelude to cell activation. Although its exact role in modulating T cell signaling is unknown, it is commonly believed that the immunological synapse is the site of cross-talk between the T cell and APC (or target). We have examined the synapses formed by naive and memory CD4 cells during Ag-specific cognate interactions with APCs. We show that the mature immunological synapse forms more quickly during memory T cell activation. We further show that the composition of the synapse found in naive or memory cell conjugates with APCs is distinct with the tyrosine phosphatase, CD45, being a more integral component of the mature synapses formed by memory cells. Finally, we show that signaling molecules, including CD45, are preassociated in discrete, lipid-raft microdomains in resting memory cells but not in naive cells. Thus, enhanced memory cell responses may be due to intrinsic properties of signaling molecule organization. *The Journal of Immunology, 2004, 173: 33–41.

During an infection, T cells are induced into clonal expansion followed by differentiation into effector and memory cells. In both primary and secondary immune responses T cell activation, following engagement of TCRs by MHC-peptide complexes involves multiple signal transduction pathways. Cell signaling is tightly regulated and requires the organization of individual signaling molecules into discrete complexes both on the T cell membrane (e.g., lipid raft microdomains) (1–3) and between the T cell and APC during cell-cell contact (e.g., immunological synapses) (4–6). Discrete localization of interactive molecules may both control signal intensity and duration by clustering or separating important activating or inhibitory molecules.

In the immunological synapse, relevant interactive molecules are found in supramolecular activation clusters (SMACs) with TCRs and their MHC-peptide ligands, and associated signal transduction molecules, localized to the center of contact (c-SMAC) surrounded by a peripheral ring (p-SMAC) of adhesion and regulatory molecules (7). This “immature” immunological synapse, as recent studies have shown that T cell and APC adhesive molecules make initial contact and initially organize centrally, with signaling molecules located at the periphery of the T cell-APC junction (8). Thus, the c-SMAC and p-SMAC of the immature and mature synapses are mirror images.

Lipid rafts (glycolipid-enriched membranes or detergent-insoluble glycosphingolipid-enriched domains) are regions of the plasma membrane that possess a high content of cholesterol and sphingolipid (1–3). Proteins that possess saturated acyl chains are generally associated with lipid rafts. A number of important signal transduction molecules are constitutively associated with lipid rafts or are recruited to lipid rafts upon cell activation (9–14). Thus, the microenvironment of lipid rafts may facilitate stable interactions between signaling molecules and may also restrict contact with inhibitory proteins (2, 3, 13, 15, 16).

The major T lymphocyte involved in secondary immune responses is the memory cell. Memory CD4 T cells promote more robust immunity than do naive cells because they respond to Ag more rapidly and promote a more vigorous effector response as compared with naive cells (reviewed in Ref. 17–19). This rapid response may be attributed to a lower signaling threshold for activation and/or lesser dependence on costimulation signals (20). Further, the TCR-mediated signal transduction pathways used by memory cells may differ from pathways used by naive cells (21, 22). Although several studies have assessed the molecular organization of signal transduction molecules, including immunological synapse formation, in either primary naive CD4 cells or in cloned T cell lines, less is known about the organization of signaling molecules in memory cells involved in recall immune responses. Of note, it is unclear whether immunological synapse formation and subsequent cell activation are similar during cognate interactions between naive or memory T cells and APCs. In the present study we have investigated the formation and composition of immunological synapses when naive or memory cells encounter peptide Ag. We show that immunological synapses form more quickly and are maintained longer when memory cells engage APCs than when naive T cells engage APCs. We also show that the composition of the synapse differs in memory vs naive cells. Finally, through the study of lipid raft-associated molecules, we suggest that altered signal transduction in memory cells may be due to distinct relationships between important signaling molecules. In particular, we find that the basal association of CD45 and the TCR with lipid rafts differs between memory and naive cells. Hence, our findings suggest that robust memory cell activation might be facilitated by distinct structural organization of critical signal transduction elements.
Materials and Methods

Animals

The BALB/c ByJ and DO11.10 (23) mice used in these experiments were bred and maintained at the Wadsworth Center Animal Facility under specific pathogen-free conditions. The majority of T cells in the DO11.10 mice bear the TCR that recognizes a chicken OVA-derived peptide, OVA253-269 (hereafter referred to as OVA), presented by I-<sup>A</sup>-<sup>2</sup> (23). This TCR is encoded by transgenes encoding V<sub>α</sub>2,8.2/Nr13.1 chains and can be identified by the anti-clonotypic mAb, KJ1-26 (24). The experiments were performed using 10- to 12-wk-old mice. Both male and female mice were used in different experiments with no discernible differences in the results. All mice used in these studies were maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (Washington, DC). All experiments were approved by the Wadsworth Center Institutional Animal Care and Use Committee.

Reagents and Abs

mAbs KJ1-26 (anti-DO11.10 clonotype) (24), C363.29B (anti-CD3e) (25), 23G2 (anti-CD45RB) (26), and M1/9,3HL.2 (anti-CD45) (27) were prepared from the supernatants of hybridoma cell lines. Abs directed against mouse CD11a (LFA-1), protein kinase C (PKC)-<sup>θ</sup>, linker for activation of T cells (LAT), and CD71 (Santa Cruz Biotechnology, Santa Cruz, CA); Lck and ZAP-70 (Upstate Biotechnology, Charlottesville, VA); cholera toxin-B (CTX-B) rhodamine-conjugate (List Biological Laboratories, Campbell, CA); anti-phospho-ZAP-70 (Try319; Cell Signaling Technology, Beverly, MA); and anti-CTX-B subunit (Calbiochem-Novabiochem, San Diego, CA) were purchased. CFSE and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine peroxidase (DiD) were purchased from Molecular Probes (Eugene, OR). Methyl β-cyclodextrin (MβCD) was purchased from Sigma-Aldrich (St. Louis, MO). The Ab directed against phosphorylated Lck (pY394) was obtained from A. Shaw (Washington University, St. Louis, MO). Primary Abs were either directly conjugated to Alexa Fluor 488, 568, or 647 using Ab labeling kits or were detected with secondary Alexa Fluor 488, 568, or 647 Abs (Molecular Probes).

Preparation of cells

In all experiments, nonimmune mice were used, and enriched populations of CD4 T cells were prepared by negative selection procedures as previously described (28) and were 90–95% CD4<sup>+</sup> and <3% surface Ig<sup>+</sup> as determined by flow cytometry. Naive and memory cell populations were separated based upon CD45RB expression using mAb 23G2 and MACS (Miltenyi Biotec, Auburn, CA) sorting to separate the CD45RB<sup>high</sup> (naive) and CD45RB<sup>low</sup> (memory) cells. Following separation, the sorting mAb was removed using a low pH buffer as described. APCs were prepared by depletion of T cells from populations of splenocytes, as described (29).

Conjugate formation and immunofluorescence

Conjugates between T cells and APCs were formed by mixing T cells and OVA-pulsed (1.5 μg/ml) APCs at a 1:2 ratio and a quick centrifugation at 400 × g to initiate cell-cell contact. Cells were incubated at 37°C, 5% CO<sub>2</sub> for various durations. For measurement of conjugate formation, T cells were labeled with CFSE and APCs were labeled with DiD before mixing. Following incubation, cultured T cell/APC conjugates were analyzed by flow cytometry to determine the percentage of T cells that had both CFSE and DiD staining, indicating a conjugation of CFSE-labeled T cells with DiD-stained APCs.

For immunofluorescence studies of T cell-APC conjugates, following incubation, the cells were fixed with freshly prepared 4% paraformaldehyde for 20 min at room temperature and allowed to adhere to poly-L-lysine coated slides at 4°C overnight or 2 h at 37°C (8). Cells were permeabilized with 0.2% Triton X-100, blocked with 1% BSA in PBS and stained with respective Abs for 1 h diluted in 1% BSA/PBS. Cells were washed four times with PBS between primary and secondary Abs and before addition of mounting solution. Coverslips were mounted onto slides using SlowFade Light Antifade kit (Molecular Probes) following the manufacturer’s specifications.

In experiments in which lipid rafts and raf-associated proteins were examined, CTX-B rhodamine was used to label the endogenous GM1 glycosphingolipids of the T cells before mixing with APCs (30). Disruption of lipid rafts was accomplished by treating the T cells with 10 mM MβCD for 30 min at 37°C (31). Preliminary experiments determined that these treatment conditions did not result in loss of cell viability and, further, within several hours after MβCD removal, cellular cholesterol content was similar to that found in untreated controls. Aggregation of lipid rafts, or patching, was induced in unstimulated cells by incubating the cells with anti-CTX-B Ab (1/250 in PBS/0.1% BSA; Calbiochem-Novabiochem) for 30 min on ice, and then 20 min at 37°C (30). For stimulated cells, CTX-B rhodamine-labeled T cells were mixed with OVA peptide-pulsed APCs at a 1:2 ratio and quickly centrifuged at 400 × g. Cells were incubated at 37°C, 5% CO<sub>2</sub> for 1 h at 37°C. Slides were washed four times with PBS in between primary and secondary Ab incubations and before the addition of mounting solution. Coverslips were mounted as previously discussed. Confocal microscopy images were acquired with an OZ Confocal system (Noran Instruments, Middleton, WI) attached to an Olympus IX70 microscope. Differential interference contrast (DIC) images were acquired with a digital camera attached to the Olympus IX70 microscope. Three-dimensional reconstruction of the T cell/APC interface was generated with 0.3 μm serial sections of z-axis images along the z-axis and subsequent analysis with Intervision software (Noran Instruments).

Results

Conjugation of APCs with naive or memory CD4<sup>+</sup> T cells

To study Ag-specific cognate interactions, primary naive and memory CD4<sup>+</sup> T cells were prepared from the spleens of DO11.10 mice (23). The TCRs expressed on these CD4<sup>+</sup> cells specifically bind to the peptide OVA<sub>253-269</sub> presented by the class II MHC molecule, I-A<sup>α</sup>-A<sup>α</sup> (23). The transgenic TCR is specifically recognized by the anti-clonotypic mAb, KJ1-26 (24). We have previously reported on the generation and characterization of memory cells that bear the clonotypic TCR in DO11.10 mice (29, 33, 34). Of note, KJ1-26<sup>+</sup>, OVA-specific memory T cells were obtained from unmanipulated mice. These cells arise due to original stimulation through a dual-expressed TCR composed of the transgenic TCR β-chain paired with an endogenous TCR Vα-chain (29). As previously discussed, these cells are typical memory CD4<sup>+</sup> cells (34). In the current study, we evaluated immunological synapses formed during naive vs memory cell cognate interactions with APCs. We assessed T cell to APC conjugates formed after coincubation (8). In initial experiments, we determined whether naive and memory cells were comparable in their ability to interact with APCs. Conjugate formation was analyzed by flow cytometry using lipophilic dyes to track both the APCs (DiD) and the T cells (CFSE). The T cells were added into culture with OVA-pulsed APCs and subsequently analyzed by flow cytometry to evaluate cell conjugates formed displaying both DiD and CFSE fluorescence. In the absence of peptide, no conjugates were observed (Fig. 1). In contrast, peptide-pulsed APCs effectively interacted with both naive and memory T cells. As indicated, neither T cell type preferentially interacted with APCs as comparable frequencies (~25% of total input T cells at most time points) of conjugates were formed by either naive or memory T cells (Fig. 1).

Formation of immunological synapses formed by naive and memory CD4<sup>+</sup> T cells

Discrete stages of cell signaling were identified at different times after T cell-APC contact by immunolocalization of the TCR and representative adhesion (LFA-1) or signaling (PKC-θ) molecules. Previous studies showed that in primary naive CD4<sup>+</sup> T cells both the TCR and PKC-θ are found in the c-SMAC of the mature immunological synapse after initial localization to the p-SMAC (7, 8, 31). In contrast, LFA-1 is restricted to the p-SMAC of the mature synapse after initial localization to the c-SMAC. Using these markers, we have identified the p-SMAC and c-SMAC in both the immature and mature immunological synapses of naive cells (Fig. 31). In contrast, LFA-1 is restricted to the p-SMAC of the mature synapse after initial localization to the c-SMAC. Using these markers, we have identified the p-SMAC and c-SMAC in both the immature and mature immunological synapses of naive cells (Fig. 31).
2A). For convenience, and because several of the subsequent figures depict TCR localization, but do not always show LFA-1, we describe the stage of the immunological synapse with respect to TCR localization. Thus, our usage of the term immature synapse applies to conjugates in which the majority of the TCR flanks the central contact point, c-SMAC, between the T cell and APC. In this case, the TCR is largely associated with the p-SMAC. We use mature synapse to describe the conjugate stage in which the major portion of the TCR is associated with the c-SMAC. Although not always shown, LFA-1 was examined in each experiment and is reproducibly found with a reciprocal localization to the TCR (e.g., in Fig. 2). It should be noted, however, that protein localization in the immunological synapse is dynamic, so that a temporal analysis of mature synapse formation and dissolution shows points of redistribution of proteins (e.g., LFA-1, TCR) from the p-SMAC to the c-SMAC (and vice-versa) with a greater or lesser amount found in the c-SMAC at a given time point.

In the current study, both naive and memory CD4 T cell conjugates with OVA-pulsed APCs were examined. As previously shown, in conjugates containing naive cells, PKC-θ/H9258 and TCR were found in the p-SMAC with LFA-1 found in the c-SMAC early after initial cell-cell contact (5–15 min) (Fig. 2A, x-y images not shown). However, by 30 min the pattern had reversed with TCR and PKC-θ localized in the c-SMAC surrounded by LFA-1 in the

FIGURE 1. Equal percentages of conjugates are formed between APCs and naive or memory T cells. Naive (■) and memory (□) DO11.10 CD4+ T cells were labeled with CFSE. Splenic APCs were labeled with DiD and loaded with 1.0 μg/ml OVA323–339. T cells and APCs were quickly mixed and conjugated by a brief centrifugation step. They were then incubated at 37°C for the indicated lengths of time. The cultured cells were analyzed by flow cytometry, and the percentage of cells forming conjugates (both CFSE and DiD staining) was calculated.

FIGURE 2. Maturation of the immunological synapse of naive and memory CD4 T cells. DO11.10 TCR transgenic T cells were isolated, separated into naive and memory cell populations based on CD45RB expression, and conjugated with OVA323–339 loaded splenic APCs from BALB/c mice. At the indicated time points the cells were fixed, permeabilized, and stained with Abs to TCR, PKC-θ, and CD11a (LFA-1). Data for Ab to the transgenic TCR (KJ1-26) is shown although we observed similar results with either anti-CD3 or pan-specific anti-TCR Abs. Conjugates were analyzed by confocal microscopy. a, Naive or memory T cells indicating TCR (red), PKC-θ (green), and LFA-1 (blue) staining with three-dimensional reconstruction along the x-z-axis. The far right column shows the overlays. b, Shown are the percentages of conjugates containing either naive (■) or memory (□) cells with PKC-θ and TCR in the c-SMAC and LFA-1 in the p-SMAC. Data is from three separate experiments, examining a minimum of 40 conjugates per experiment; * = p < 0.02; ** = p < 0.002, based on the paired t test.
Once formed, the mature synapse was relatively short-lived, as by 60–90 min we observed a redistribution of the molecules and a down-regulation of TCR expression. The down-regulation of TCR expression has been noted previously and likely is a consequence of TCR internalization (8, 35). Based on the three indicator molecules, we found that similar immunological synapses formed between memory cells and Ag-pulsed APCs. However, as compared with naive cells, mature synapses formed by memory cells occurred much more quickly and were more stable. Hence, whereas naive cells formed mature synapses by 30 min, memory cells formed mature synapses within 5–15 min (Fig. 2, B and C). Further, we did not observe redistribution of membrane molecules or TCR internalization until after 90 min of conjugate formation (data not shown).

Activation of signal transduction molecules during formation of the naive or memory CD4 T cell immunological synapse

TCR-mediated signal transduction is associated with the formation of a productive immunological synapse (4–6). As indicated by the general induction of tyrosine phosphorylation, we confirmed a previous study (8) that in naive T cells signaling begins at the periphery of the immature synapse with the subsequent migration of tyrosine phospho-proteins to the c-SMAC of the mature synapse, followed by diminishing signaling (phospho-tyrosine immunostaining intensity; data not shown). We also observed that signaling events occurred similarly in memory T cells except that, as noted in Fig. 2, a faster formation of mature synapses led to earlier migration of tyrosine phospho-proteins to the c-SMAC (data not shown). TCR-mediated signal transduction in naive and memory T cells was best illustrated through examination of two critical TCR-regulated cytoplasmic tyrosine kinases, Lck and ZAP-70. A recent study of naive T cells indicated that Lck is activated in the p-SMAC of the immature synapse and signaling is terminated before synapse maturation, whereas activation of its substrate, ZAP-70, occurs in the c-SMAC of the mature synapse (8). Hence, we assessed activation of both kinases by immunostaining with site-specific Abs directed against key phospho-tyrosine residues in Lck and Zap-70 (Y394 and Y319, respectively). Cell conjugates were formed between APCs and either naive or memory T cells followed, at various times, by fixation and immunostaining with Abs to the TCR, anti-pY394, and Lck protein. Subsequent to synapse formation, Lck protein (active or nonactive) was found associated with the TCR either in the immature or mature immunological synapse (Fig. 3A, x-y images not shown). As the synapse dissolved (60 min), Lck dispersed from the contact area and became redistributed around the T cell (data not shown). In contrast to Lck

**FIGURE 3.** Activation of Lck and ZAP-70 in the immunological synapses of naive and memory T cells. DO11.10 TCR transgenic naive memory T cells were isolated and conjugated with OVA323–339 loaded splenic APCs. At the indicated time points the cells were fixed, permeabilized, and stained with Abs to (a) TCR (red), active Lck (pLck, green), and Lck (blue), or to (b) TCR (red), active ZAP-70 (pZAP-70, green), and ZAP-70 (blue). Shown is staining with three-dimensional reconstruction along the x-c-axis and the far right columns show the overlays. c, Shown are the percentages of conjugates containing naive (■) or memory (□) cell with pLck or pZAP-70 in the c-SMAC. Data are from three separate experiments, examining a minimum of 40 conjugates per experiment; * = p < 0.02; ** = p < 0.002, based on the paired t test.
protein, active Lck displayed a more regulated pattern of localization. At early time points (5 min; Fig. 3A) active Lck colocalized with the TCR in the p-SMAC of the immature synapse. By 15 min after conjugate formation, the amount of active Lck had decreased and was undetectable when the mature synapse was formed (30 min; Fig. 3C). Thus, although Lck associated with the TCR in the c-SMAC of the mature synapse, it was no longer involved in TCR-mediated signaling. This contrasts to the overall pattern of Lck activation in memory cells. Although we did observe that the Lck protein was also associated with the TCR in memory cells throughout synapse formation, we found that active Lck was present in the c-SMAC of the mature synapse (Fig. 3A). Lck-mediated signaling not only began quickly, but it was also prolonged in memory, as compared with naive, lasting up to 30 min after conjugate formation (Fig. 3C).

One consequence of Lck-mediated signaling is the enzymatic activation of one of its substrates, ZAP-70 (36, 37). Thus, downstream events in TCR signaling were studied through examination of ZAP-70 activation. Conjugates were formed between APCs and either naive or memory CD4 T cells. The cells were fixed and immunostained with Abs to TCR, active ZAP-70 (anti-pY319), and ZAP-70 protein. In both naive and memory T cells ZAP-70 behaved as Lck, and was found with the TCR throughout synapse formation (Fig. 3B). However, unlike phospho-Lck, active ZAP-70 was observed in both the immature and mature synapses of naive and memory cells. Specifically, we found active ZAP-70 as early as 5 min and as late as 60 min after initial conjugate formation. This pattern was similar in naive and memory T cells (Fig. 3B).

Because the mature synapses formed more quickly in memory cells, ZAP-70 migrated more rapidly to the c-SMAC in memory cells. However, activation of ZAP-70 occurred with similar kinetics and lasted for comparable periods in both naive and memory cells (Fig. 3C). Hence, even though Lck-mediated signaling occurred for a longer time period in memory cells, its effect on at least one substrate, ZAP-70, was not prolonged.

Localization of CD45 in naive or memory CD4 T cell immunological synapses

The transmembrane tyrosine phosphatase CD45 plays an essential role in T cell mediated signal transduction likely due to its ability to promote dephosphorylation of the inhibitory carboxy-terminal tyrosine of Lck (38). Such dephosphorylation leads to or maintains Lck activation and subsequent phosphorylation of the TCR complex and ZAP-70 (39). Because we observed a difference in Lck activity between naïve vs memory cells, we determined whether a differential association of CD45 with the immunological synapse might influence Lck activation. Previous studies have shown that CD45 was restricted to the p-SMAC of the mature synapse in which it was spatially separate from the TCR (40, 41). Indeed, we initially identified CD45 in the p-SMAC of conjugates between APCs and naïve CD4 T cells (Fig. 4A). Although we examined conjugates at multiple time points, we did not observe the transient association of CD45 with the c-SMAC of the mature synapse (data not shown). However, further study showed that CD45 was actually localized to a distinct region of the synapse outside the c-SMAC but separate from LFA-1 (Fig. 4A). Thus, these two p-SMAC-associated molecules exhibit a fixed higher level ordering in relation to the c-SMAC within the mature synapse with LFA-1 being the more distal.

We next examined the structure of the immunological synapses formed between OVA-pulsed APCs and memory CD4 T cells. In contrast to those formed by naive cells, the synapses formed by memory cells were unique with respect to CD45. Upon conjugate formation and throughout the cognate interaction, CD45 was found associated with the TCR (Fig. 4B). Thus, when the mature synapse formed, CD45 was in the c-SMAC surrounded by the p-SMAC (identified by LFA-1 staining in Fig. 4B). Such colocalization of the TCR and CD45 is more easily seen in computer reconstructions of the mature synapse along the z-axis (Fig. 4C). It is evident that in naïve cells CD45 surrounds the TCR in the mature synapse but these two molecules are not in contact. In memory cells, CD45 and TCR were colocalized in both the immature and mature synapse.

Composition of lipid rafts in naïve and memory CD4 T cells

T cell activation and signal transduction are dependent upon the rearrangement of lipid rafts. Several molecules necessary for T cell activation and signaling are constitutively attached or migrate to lipid raft compartments thus creating signaling platforms to facilitate interactions or to segregate regulatory elements. Because lipid raft microdomains are relatively resistant to extraction by nonionic detergents, such as Triton X-100, raft-associated molecules are
typically identified after separation of membrane detergent lysates on sucrose gradients (42, 43). However, large numbers of cells are required for this technique, hence, we instead identified raft-associated proteins on single cells using microscopy (30, 32). Single cells or T cell to APC conjugates were exposed to Triton X-100 and nonraft-associated molecules were extracted from cells. Previous studies have shown that after detergent solubilization, 80–90% of total membrane lipid rafts remain (44). Lipid rafts may be visualized by fluorescent microscopy after labeling with fluorescent CTX-B subunit, which binds to GM1 gangliosides (45, 46). For example, biochemical studies have demonstrated that the T cell signal transduction molecule, LAT, is constitutively associated with lipid rafts whereas the transferrin receptor (CD71) is not associated with rafts (10, 42, 47). When naive or memory T cells were examined by microscopy, both LAT and CD71 immunostaining was apparent (Fig. 5). However, when either cell type was exposed to 1% Triton X-100 so that nonlipid raft components were extracted, only lipid rafts (CTX-B staining) and raft-associated molecules (e.g., LAT) remained.

We next analyzed the structure of lipid rafts on naive and memory cells with respect to the presence of the TCR and associated signaling molecules. Although similar patterns were observed with respect to lipid raft association of several signaling molecules (e.g., Lck, ZAP-70; our unpublished observations), a striking difference was seen in the relationship of both TCR and CD45 with lipid rafts. Before Ag-mediated activation, neither CD45 nor TCR were associated with lipid rafts in naive cells (Fig. 5). When naive or memory T cells were examined by microscopy, both LAT and CD71 immunostaining was apparent (Fig. 5). However, when either cell type was exposed to 1% Triton X-100 so that nonlipid raft components were extracted, only lipid rafts (CTX-B staining) and raft-associated molecules (e.g., LAT) remained.

We also examined lipid rafts during cognate interactions between OVA-pulsed APCs and naive or memory CD4 + T cells. In these experiments, the T cells were labeled with CTX-B rhodamine before conjugate formation. Conjugates were treated with or without Triton X-100 before immunostaining and fluorescence microscopy. In general, lipid rafts were organized in concert with the TCR as rafts were initially localized in the p-SMAC of the immature immunological synapse before migrating to the c-SMAC of the mature synapse. Further, in conjugates containing naive cells, the TCR became associated with lipid rafts, acquiring resistance to detergent extraction (Fig. 7A). In contrast, CD45 remained distinct from lipid rafts and detergent soluble throughout the naive cognate interaction. Finally, in conjugates containing memory T cells, we found that both the TCR and CD45 remained detergent soluble (Fig. 7B), strongly suggesting that these two molecules remain associated with lipid rafts throughout the cell activation process.

**Discussion**

In this report we describe the organization of the immunological synapse formed between APCs and memory CD4 + T cells. The majority of previous reports examining the formation and composition of the immunological synapse have focused on synapses formed by naive CD4 or CD8 T cells or thymocytes. We began this study to determine whether memory CD4 + T cells formed similar organizational platforms when they came in contact with APCs presenting cognate Ag. Because the DO11.10 mouse is a source of Ag-specific naive and memory CD4 + T cells, we could make precise comparisons of the two cell types during early cell activation.

Study of immunological synapses formed by naive and memory T cells demonstrated several differences. Key among these were the more rapid and stable formation of the mature synapse in memory, as compared with naive cells. Whereas naive cells did not form mature synapses until nearly 30 min after conjugation with APCs, memory cells formed mature synapses by 5–15 min. Further, mature synapses could be detected up to 90 min after initial formation in memory cells, suggesting that TCR down-regulation, previously described for naive cells (8), did not occur as readily in memory cells. The formation of immunological synapses in naive cells requires costimulation-driven active transport (49). Thus, the more rapid synapse formation by memory T cells is consistent with 

**FIGURE 5.** Lipid raft composition of naive and memory T cells. DO11.10 TCR transgenic T cells were isolated and separated into naive (a and c) and memory (b and d) cell populations and labeled with CTX-B subunit–rhodamine to identify lipid rafts (GM1). In each figure the cells were either (control) fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and then stained with fluorescent-labeled antibodies or were (1% TX-100) permeabilized with 1% Triton X-100, fixed with 4% paraformaldehyde, and then stained with fluorescent-labeled Abs. a and b, Unstimulated naive and memory T cells were stained with Abs to GM1 (red), LAT (green), and CD71 (blue). c and d, Unstimulated naive and memory T cells were stained with Abs to GM1 (red), TCR (green), and CD45 (blue). Overlays and DIC images of the individual T cells are shown in the far right columns.
reports indicating that memory cells are less dependent upon co-stimulation (20). It is also interesting to note another study showing that naive cells, which had been activated with Ag and IL-2 before conjugate formation, also rapidly formed immunological synapses (40). Thus, we suggest that the ability to rapidly form synapses is acquired early in the transition from resting naive cells into activated or effector T cells and is a property maintained by resting memory cells.

It is generally believed that formation of a complete synapse is critical to productive signaling and T cell activation (50). For example, a mature, organized immunological synapse forms when agonist but not antagonist peptides mediate the cognate T cell interaction (7, 51). However, the view that a mature synapse is required for signaling has been recently challenged by reports of productive signaling in the p-SMAC of the mature synapse (8). Certainly in naive T cells immediate signal transduction, as demonstrated by activation of Lck, begins and ends before migration of the src kinase/TCR to the c-SMAC (Fig. 3A). However, in conjugates containing memory T cells, mature synapses are formed so rapidly that Lck-mediated signaling occurs in the mature c-SMAC. Further, Lck-mediated signaling is prolonged in memory T cells. Still, despite the centralized and prolonged activation of Lck, the duration of activation of its immediate downstream substrate, ZAP-70, was similar in both naive and memory CD4 cells. Hence, our data suggest that downstream signaling is not more robust in memory cells. At present, we cannot rule out the possibility that Lck may promote alternative, memory cell-specific, signaling pathways and that the longer period of Lck activation in memory cells may facilitate such alternative signaling. We also note that we did not observe discrete stages of ZAP-70 activation as reported by Freiberg et al. (40). We suggest that this discrepancy may be related to a difference in the activation state of the cell populations used in our study (resting primary cells) vs their study (preactivated cells). An alternative explanation for our failure to identify different stages of ZAP-70 activation may be that the phospho-specific anti-ZAP-70 mAb used in our study recognizes only a single, activation-specific phospho-tyrosine (pY319) on the active kinase, whereas in Freiberg et al. (40) a mAb was used that recognizes two phospho-tyrosines (pY493 and pY492). We cannot exclude the possibility that different sites on ZAP-70 are phosphorylated at different points during T cell activation.

The tyrosine phosphatase, CD45, is typically described as a protein that is excluded from the c-SMAC of immunological synapses formed by mature CD4 or CD8 T cells and thymocytes. In this report, we show that a primary difference in synapses formed by memory, as compared with naive, CD4 T cells is the localization

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**FIGURE 6.** Disruption of lipid rafts with MβCD. a and c, Naive and memory (b and d) DO11.10 T cells were labeled with CTX-B subunit-rhodamine to identify lipid rafts (GM1). The cells were left untreated or treated with 10 mM MβCD for 30 min at 37°C. In each figure the cells were either (control) fixed with 4% paraformaldehyde, permeablized with 0.2% Triton X-100, and then stained with fluorescent-labeled Abs or were (1% TX-100) permeablized with 1% Triton X-100, fixed with 4% paraformaldehyde, and then stained with fluorescent-labeled Abs. a and b, Unstimulated naive and memory T cells were stained with Abs to GM1 (red), LAT (green), and CD71 (blue). c and d, Unstimulated naive and memory T cells were stained with Abs to GM1 (red), TCR (green), and CD45 (blue). Overlays and DIC images of the individual T cells are shown in the far right columns.
of CD45 to the c-SMAC of memory cells vs the p-SMAC of naive cells. Importantly, in naive cells CD45 is spatially separated from the TCR-Lck complex, whereas it colocalizes with the TCR in memory cells. This observation is reminiscent of previous studies that CD45 could be coimmunoprecipitated with the TCR and CD4 localization in the mature synapses formed by both naive and memory T cells, in which it is found extending from the c-SMAC into the abutting portions of the p-SMAC (data not shown). Hence, in naive cells, whereas CD45 and the TCR are not associated, CD4 colocalizes with both molecules, whereas in memory cells the TCR, CD4, and CD45 colocalize. The functional consequences of this differential location of CD45 are unclear at present. However, it is tempting to speculate that alternative regulation of signaling in memory cells results from the colocalization of the TCR and CD45 within the synapse. Such colocalization of CD45 and the TCR may allow for more efficient dephosphorylation of the inhibitory tyrosine residue of Lck to increase or lengthen the degree of Lck activation. Correspondingly, we did find a longer period of Lck activation in conjugates between memory cells and APCs. However, Lck kinase activity was not higher in memory cells, as compared with naive cells (data not shown). This may reflect contributions of CD45 to dephosphorylation of the positive regulatory tyrosine residue of Lck or to alternative regulatory modifiers, such as CTLA-4 or SHP-1.

The composition of membrane microdomains, such as those containing lipid rafts, further distinguishes naive and memory T cells. Lipid rafts have recently been shown to accumulate in the c-SMAC after movement from the peripheral zone of the synapse (53). In this study, we confirm this finding and further show a unique constitutive association of both the TCR and CD45 with lipid rafts of memory cells. Surprisingly, we report that all of the memory cell TCRs were found in the synapse. Because DO11.10 memory cells coexpress alternate TCRs to varying degrees (29, 33, 34), this suggests that both occupied and nonengaged (non-OVA-specific) TCRs migrate to the synapse. This may be related to their constitutive association with lipid rafts. In general, movement of nonoccupied TCRs to the synapse might facilitate sustained signaling by increasing their proximity to MHC-peptide complexes. In contrast, in naive cells the TCR became strongly associated with lipid rafts only upon activation. Again, we find that all of the TCRs localize to the immunological synapse. Also, in naive cells, CD45 was excluded from lipid rafts in both resting cells and throughout the conjugate (activation) process. Interestingly, another recent report indicated that a small amount (~5%) of CD45 could be identified in the raft-associated fraction after sucrose gradient centrifugation (54). Thus, if this is similar in our experimental model, we can set a detection level of >5% to our microscopy analysis. However, we also note that the study examined T lymphoma cells and thymocytes (54). Hence, it is possible that CD45 is completely excluded from the membrane rafts in naive cells during initial activation.

As with its location in the immunological synapse, the constitutive and stable association of CD45 with lipid rafts in memory CD4 T cells suggests that signaling in memory cells is unique. Again, there is opportunity for CD45 and Lck to have a more stable association in memory cells that might directly contribute to the observed prolonged Lck activation. Alternatively, it is possible that CD45 is better able to affect other raft-associated regulatory elements, such as the nonreceptor tyrosine kinase Csk. At present it is not clear as to why the localization of CD45 in either membrane domains or during synapse formation is distinct in naive and memory cells, however, the difference is likely due to the isoform of CD45 present on either cell type. Although naive and memory CD45 isoforms differ in the extracellular portion of the molecule, intermolecular associations with other surface molecules might influence where CD45 localizes. Interestingly, one report suggests that CD45 localization on certain cloned Th cell lines might differ (55). Specifically, there is evidence that CD45 associates with lipid rafts in Th1 cell membranes but not in Th2 cell membranes (56). Interestingly, the isoform of CD45 found used by Th1 cells is the same as that found on memory cells (CD45RO), whereas both naive cells and Th2 cells are strongly recognized by anti-CD45RB Abs (56). Whether there are also differences between Th1 and Th2 cells with respect to immunological synapse formation, especially with respect to the localization of CD45 is unclear.

How the distinct immunological synapse and membrane microdomain associations might contribute to faster and more robust memory cell responses has not yet been determined. We and others (21) have observed that memory cells can use distinct TCR-mediated signal transduction pathways in response to nonpeptide stimuli (A.R.O. Watson, and W.T. Lee, manuscript in preparation). Such differences in signal transduction between naive and memory cells may be due, in part, to the differential localization of important regulatory proteins. Further, memory cells require less TCR stimulation and less costimulation to respond to peptide than do naive cells and it is important to determine whether this might be due to a different organizational structure of the relevant signaling molecules. Other studies have suggested various roles for both the immunological synapse and the organization of proteins within lipid rafts (57). For example, it has been suggested that the primary importance of the immunological synapse lies in functions ranging from mediating polarized cytokine secretion (57), to TCR-internalization and signal termination (8), to the ordered assembly of additional signaling platforms (6). We would now propose that
immunological synapse formation also plays a primary role in facilitating memory cell function and in mediating a more efficient response to recall Ag.

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