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Single-molecule investigations of T-cell activation

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Abstract

T-cell activation is the central event governing its development, differentiation, and effector functions. T-cell activation is initiated by the direct physical interaction of the T cell antigen receptor (TCR) with cognate peptide presented by the major histocompatibility complex (pMHC) molecule expressed on the antigen presenting cell (APC) surface. Since the identification of TCR as the only receptor for antigen on T cells three decades ago, studies have elucidated the major molecular players and signaling events responding to TCR stimulation. However, the question of how the physical event of pMHC binding is converted across the membrane into chemical events to initiate signal transduction remains elusive. Here we review recent investigations of T-cell activation using single-molecule force and fluorescence techniques that shed new light on this key question.

Introduction

αβ T cells are the sentinel players of the cellular adaptive immunity, combating foreign pathogens and cancer cells. Using the T cell receptor (TCR), in conjunction with the coreceptor CD4 or CD8, they recognize antigenic peptides presented by the respective major histocompatibility complex (pMHC) class II or class I molecules expressed on antigen presenting cells (APCs), which provide the primary signals to determine T-cell activation and effector functions (Fig. 1A) [1]. Studies using traditional approaches have identified a list of players and a series of events during T-cell activation [2]: 1) TCR triggering by cognate pMHC induces the phosphorylation of CD3 immunoreceptor tyrosine-based activating motifs (ITAMs) by Src family kinase Lymphocyte-specific protein tyrosine kinase (Lck) and/or Fyn, 2) recruitment of Zeta-chain-associated protein kinase 70 (Zap70) and its activation by Lck, 3) phosphorylation of linker for activation of T cell (LAT) by Zap70 and further signaling propagation into multiple pathways, 4) formation of an immunological

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synapse (IS) between the T cell and APC where molecular organization undergoes large dynamic changes, and 5) activation of transcriptional factors and the subsequent gene expression (Fig. 1B). These findings have established the conceptual framework of our understanding of T-cell activation, allowing us to explore each step in more detail at the single-molecule level. In this review, we will summarize the recent findings of T-cell activation empowered by various single-molecule techniques and discuss the biological implications and future directions. We will focus on studies of mechanical forces in T-cell activation in response to the growing interest in T-cell mechanobiology.

Single-molecule TCR–pMHC interaction kinetics

The affinity and kinetics between TCR and cognate pMHC have been a focus of study for a long time, as they are key determinants of antigen recognition, discrimination, and T-cell activation. Early studies of bulk molecules in solution (i.e., three-dimension, or 3D measurements) using surface plasma resonance (SPR) and isothermal titration calorimetry (ITC) provided the first characterization and categorized the TCR–pMHC interaction as weak with μM K_D and a fast dissociation rate [3,4]. Comparing the affinities and kinetic rates of the same TCR interacting with a panel of altered peptide ligands (APLs) revealed that differences among pMHCs with a large functional range are small, and their correlation with function lacks consistency, making 3D affinity and kinetic rates inadequate parameters for explaining antigen discrimination.

Using micropipette adhesion frequency assay [5], more recent studies of surface-bound pMHC interacting with native TCR expressed on T cell membrane (i.e., *in situ* or 2D measurement) found that the 2D parameters have significantly larger dynamic range and better correlate with T-cell function [6-9]. Compared to their 3D counterparts, the 2D affinities for TCR interaction with agonist pMHC are strong – as strong as high affinity interactions between activated lymphocyte function-associated antigen 1 (LFA-1) and intercellular adhesion molecule 1 (ICAM-1) [10], the *in situ* dissociation is much faster – with bond lifetimes as short as ~100 ms and 2D on-rates spanning several logs. The huge range of 2D on-rate for APLs suggest that on the cell membrane, TCR–pMHC association is reaction-limited, which is physically distinct from the diffusion-limited protein-protein association process in 3D.

In situ TCR–pMHC interactions were also examined by single-molecule imaging in immunological synapse formed between T cells and lipid bilayer functionalized with cognate pMHC and ICAM-1. Molecular binding events were monitored using single-molecule Förster Resonance Energy Transfer (smFRET) or single–molecule particle tracking. In FRET-based assays, pMHC and TCR are labeled with a Cy3-Cy5 FRET pair and imaged for donor-only, acceptor-only, and acceptor-bleach fluorescence. Association and dissociation events were reported by the increases and decreases in smFRET efficiency, respectively. Monitoring smFRET changes over time revealed faster dissociation (~100 ms) and increased affinity for $CD4^+$ T cells expressing 2B4 or 5c.c7 TCR interacting with MCC:I-E^k pMHC class II, similar as that reported for $CD8^+$ OT1 T cells [11]. In particle tracking-based experiments, fluorescently labeled pMHC is imaged alternately using short- and long-exposure durations per frame. While short exposure resolves all pMHC molecules,

long-exposure only highlights immobilized pMHCs because freely diffusing ones generate dim and blurred spots. Tracking the immobilized pMHCs over time until it becomes mobile or bleached defines the duration in this trapped state, termed “dwell time”. Notably, the dwell time between 5c.c7 TCR and MCC:I-E^k is ~5 s, similar to its 3D counterpart but almost 50-fold longer than the bond half-life estimated in smFRET assay [12]. One possible explanation of the discrepant results is that the immobilized state may be caused by rebinding of the pMHC to the same TCR or one in the same cluster of TCRs. Nevertheless, the *in situ* studies underscore the importance of examining TCR–pMHC interactions in T-cell native environment and demonstrated that instead of constant rates measured in solution, TCR–pMHC interactions can be regulated by cell membrane composition, cell cytoskeleton network, and cellular signaling [9,13,14].

Force and TCR triggering

Using force techniques to study the dynamics of TCR binding, recent work has found force-dependent binding kinetics (e.g. catch bonds) [8,13,15-19], substrate stiffness sensing through the TCR [20,21], and force-induced cellular signaling [15,22-24], raising significant shared interest in mechanobiology and immunology. Since many of these observations have to be made in a tightly-controlled *in vitro* environment, a basic knowledge of the techniques and measurement methods used is required in order to appreciate the impacts of mechanical forces on single TCR–pMHC bonds. We therefore review the context in which forces apply to the TCR *in vitro* in order to relate the findings back to the cell’s physiologic experience *in vivo*.

The majority of studies of TCR mechanotransduction utilize artificial pMHC presentation on a force transducer to measure the dynamics of pMHC binding under mechanical strains in the TCR’s native cellular environment. Two sets of techniques are being used [25]. The first set applies external force to the T cell via the TCR, including biomembrane force probe (BFP) [8,13,15,17-19], atomic force microscopy (AFM) [26], optical tweezers (OT) [16,22,23], and magnetic tweezers (MT) [19]. The second set measures or restrict the T cell-generated endogenous forces on the TCR, including traction force microscopy (TFM) [27], microfabricated post-array-detectors (mPAD) [28,29], molecular tension probes (MTP) [30,31] and tension gauge tethers (TGT) [32]. In assays using the first set of techniques, the pMHC-coated surface is brought into contact with a T cell for a defined period and then retracted; from this point, a bond(s) can be stretched with different force waveforms, i.e. with controlled magnitude, duration, and direction. Perhaps the most crucial and intriguing observation has been the TCR catch bonds with agonist pMHCs observed using the force-clamp waveform, where a single TCR–pMHC bond is pulled to a given force and held until dissociation to measure how long the bond lasts, i.e. lifetime, under that force (Fig. 2A). This process is repeated hundreds of times, across many cell-probe pairs, and lifetimes at a range of forces are pooled together, binned and plotted against the applied force. Interestingly, weak agonist pMHCs have average lifetimes which expectedly decrease with force, but the average lifetimes of agonist pMHCs rise with increasing force to a point, commonly referred to as the “optimal” force where lifetime peaks, and then decrease (Fig. 2B). This phenomenon, known as catch bond, has frequently been witnessed in molecules primarily known for their roles in adhesion, such as integrins [33,34], selectins [35,36] and

cadherins [37], but has been independently verified in several human and mouse TCR systems [8,13,15-19].

The TCR catch bond behavior has been attributed to the dynamic nature of the TCR–pMHC interface upon force application, which has been observed through steered molecular dynamics simulations to increase atomic-level interactions, quantified through additional hydrogen bond formation, between the TCR CDR loops and agonist, but not non-agonist, peptides [18,19]. These force-dependent interactions have accredited to a drastic conformational change in the MHC enabling rotation and a sliding motion along the binding interface [19]. However, these studies were performed in the absence of coreceptor binding, which has long been known to enhance antigen recognition (Fig. 2C). The addition of coreceptor CD8 binding has been found to amplify ligand discrimination of thymocytes because negative selection, but not positive selection ligands were able to induce synergy between the TCR and CD8, thereby converting TCR–pMHC bimolecular slip bonds into TCR–pMHC–CD8 trimolecular catch bonds [17] termed dynamic catch [38] (Fig. 2D). The formation of dynamic catch with negative selection ligands is a dynamic process that depends on the outside-in signaling of TCR, the recruitment of CD8 via Lck, and the inside-out synergistic binding of CD8 to the pMHC pre-engaged with the TCR (Fig. 2C). The data support a mechanotransduction loop model that includes force-induced interactions among both extracellular and intracellular players to stabilize the TCR–pMHC bond as part of the loop for more durable and robust signaling [17].

Several studies have shown that mechanical simulations can significantly impact T cell signaling via the TCR [25]. The TCR was suggested to be an anisotropic mechanosensor a decade ago based on an experiment in which a T cell was placed against an anti-CD3 or pMHC-bearing bead trapped by an OT [22]. When the cell was driven to move tangentially (but not normally) relative to the bead–cell interface in a sinusoidal fashion to apply cyclic force to the TCR (and CD8), calcium fluxes were induced in T cells, indicating that mechanical stimulation on the TCR was converted into a biochemical signal. A more recent OT study found that calcium could be induced by a step displacement of the cell relative to the bead–cell interface along either tangential or normal direction, but a higher pMHC coating density was required in the latter than the former case [23]. Using a BFP to apply force on TCR normal to the bead–cell interface, calcium was induced and the calcium signal strength vs force data exhibited the same pattern as bond lifetime vs force data, increasing with force in the catch bond regime and decreasing with force in the slip bond regime [15]. This correspondence between the TCR–pMHC catch vs slip bond pattern and the T cell signaling vs not signaling pattern has been confirmed in a recent study in which other binding parameters, most noticeably affinity, were found not to correlate with signaling [18].

Remarkably, calcium was only induced when force was applied on the TCR–pMHC bond via the force-clamp waveform, but not the force-ramp waveform (despite their much larger magnitude) where a single TCR–pMHC bond was pulled at a given rate of force increase until dissociation to measure how large a force the bond sustains, i.e. rupture force, under that loading rate [15]. Interestingly, this inability for the ramp force to induce calcium was overcome by permitting the pMHC to pull both TCR and CD8 [24]. These findings are intriguing because whether catch bonds are observed may depend on the force waveform

applied to the receptor [39,40]. Even for the same TCR and pMHC pairs tested using the same BFP instrument by the same experimenter, catch bonds were not observed using the force-ramp mode [41]. It is unclear whether TCR forms catch bonds with pMHCs *in vivo* as no current technique allows *in vivo* measurement of forces on TCRs. Using the second set of techniques previously mentioned, e.g., MTP, studies found that T cells pull on their TCRs via engaged cognate pMHC with 12-19 pN forces, around the optimal forces found for catch bonds in agonist interactions [30,31]. Even in these *in vitro* assays, however, only can the magnitude but not the waveform of cell-generated forces on the TCR be measured [25].

The ability for the TCR to sense the force waveform was also revealed in the OT study where force was applied tangentially to the cell–bead interface and calcium signaling as well as cytoskeletal trafficking of the TCR were both observed [23]. When tangential force did not induce a calcium response, reversal of the force direction both induced calcium and cytoskeletal trafficking. In two studies by the Zhu lab, the calcium signal strength was found to correlate with the accumulation of sequential stimulations by multiple intermittent brief forces each of which acts on a single TCR [15,24]. These data support a kinetic proofreading mechanism with a threshold of certain amount of signaling intermediates instead of certain phosphorylation states of a single TCR, which is not required but enhanced by the accumulation of serial TCR triggering preferentially by long-lived pMHC bonds. With these studies in mind, it is becoming clear that the TCR signaling is the result of complex mechanical stimulation with dimensionality in both space and time.

In addition to their static representations, bond lifetime data also contain information about the temporal effects. The bond lifetime ensemble is pooled from repeated measurements many of which are make using the same cell over a time sequence. Measurements made earlier may induce changes in the T cell, which may impact the measurements in a later time [42]. This temporal effect must be studied in order to achieve a full understanding of the TCR–pMHC interaction dynamics, especially when considering cellular signaling responses such as those generated by the inside-out/outside-in signaling loop mentioned earlier. Additionally, a T cell *in vivo* will likely experience multiple bonds simultaneously in a physiological scenario, adding a spatial layering to the *in vivo* interaction. However, the exact spatial and temporal effects of force on TCR mechanotransduction are difficult to study and their physiological relevance is hard to assess, especially in single-molecule force experiments.

Some of these questions have been partly addressed through the use of MTP, finding that the T cell begins pulling within seconds on its cognate antigen [30]. The pulling locations also co-localized with proximal TCR signaling molecule Lck, showing overlap between the mechanotransduction apparatus and biochemical responses. These data are beginning to bridge the insights gained through single-molecule force experiments. Still significant work is required to elucidate the inner workings of TCR mechanotransduction apparatus. The question remains as to how the TCR mechanically decodes information encoded in the pMHC and translates it into a biochemical signal. Due to the structure of the TCR whose ligand-binding subunits lack signaling motifs, this mechanical information must be transferred to the CD3 subunits containing signaling ITAMs. Several models have proposed on the structural coupling and possible changes upon ligand binding on the TCR, either

through interactions between the ectodomains of TCR and CD3 [43,44] or their transmembrane segments [45]. Mechanical forces applied through engaged pMHC may induce apposition of the CD3ζζ that are spread prior to ligand engagement [46], resulting in the release the sequestered CD3 intracellular domain from the membrane to increase the availability of the ITAMs for phosphorylation and participation in downstream signaling. Combining this structural response with modifications to account for kinetics mentioned previously may shed light on how the TCR interaction layers many pieces of information to create a unique profile of the pMHC it encounters, and integrates this information into the cellular signaling machinery.

Spatial regulation

As T cell activation requires the shift of local phosphorylation state and the global signal propagation, it is crucial to understand how the key players of this signaling network (kinases, phosphatases, substrates, adaptors, etc.) are organized underneath resting T cell surface (Fig. 3A) and reorganize upon antigen stimulation (Fig. 3B). Using total internal reflection microscopy, early imaging studies uncovered the changes in molecular organization during the formation of an immunological synapse (IS) between a T cell and a functionalized lipid bilayer at the subcellular level (Fig. 3C&D). A fully established IS constitutes central supermolecular activation cluster (cSMAC) containing TCR and CD28, peripheral supermolecular activation cluster (pSMAC) containing LFA-1, and distal supermolecular activation cluster (dSMAC) containing phosphatase CD45 (Fig. 3D)[47]. Formation of such structures is largely mediated by the cytoskeleton rearrangement triggered by antigen stimulation via the TCR and involves the formation of “microclusters”[48]. It has been shown that TCR forms microclusters in periphery that function as activating sites containing Zap70 and SLP76, which move centripetally (Fig. 3C) towards the cSMAC where they are internalized (Fig. 3D) [49]. At the nanometer scale, it has been under debate whether TCR pre-clusters on resting T cell surface or only do so upon antigen stimulation (Fig. 3A). Early studies using electron microscopy reported TCR forming linear or island-like clusters on resting T cell lines and primary T cells [50,51]. This pre-clustering effect was further confirmed with super-resolution imaging techniques such as photoactivated localization microscopy (PALM) and direct stochastic optical reconstruction microscopy (dSTORM) [51-53]. However, poly-L-lysine (PLL) coated surfaces were used in many imaging studies to immobilize T cells. It is assumed to be inert to preserve the resting state of T cell, but a recent study by Santos *et al.* demonstrated the ability of PLL to induce calcium flux and TCR clustering [54]. In the study of Jung *et al.,* where cells were fixed first before immobilized on PLL surface and imaged with variable-angle TIRF and supper-resolution localization microscopy, TCRs are found to be segregated, clustering on microvilli tips [55].

Despite the controversy of the extent of TCR clustering in resting T cells, TCRs most likely functions as monomers[56,57] and forms larger clusters upon antigen stimulation[51,53]. Moreover, self-clustering and colocalization of TCR and other proximal signaling molecules represent a key spatial requirement of TCR signaling initiation and propagation. Multiple imaging studies at single-molecule scale have observed segregation of phosphatase CD45 from TCR-CD3 [58-60], resulting in shift in phosphorylation state. This is the essence of

kinetic-segregation model, which attributes TCR triggering to the physical exclusion of CD45 phosphatase, due to its large ectodomain relative to the TCR–pMHC complex (Fig. 3B). Furthermore, antigen stimulation also induces clustering of kinase Lck, and its co-localization with TCR and phosphorylated CD3[58]. Moreover, CD3 phosphorylation is more associated with TCR-CD3 clusters of high molecular density, suggesting a local threshold for sustained signaling within each cluster [53]. Further signaling to downstream requires the recruitment of Zap70 and LAT to activated TCR-CD3 clusters [51,61,62].

Future directions

Despite being a central player in T cell activation, the TCR triggering mechanism remains incompletely understood. Recent single-molecule investigations have shown that TCR signal transduction can be initiated by force, indicating that TCR is capable of mechanosensing. While mechanical forces on the TCR seems to fit naturally to the missing cause required in conformational change models, force may not be required for TCR signaling, for TCR triggering has also been observed in the absence of applied force. Regardless of whether force is involved in every and all TCR triggering events, force is likely involved in amplifying the signal transduction. The recently published TCR-CD3 complex structure has shed lights on this problem, but also raised many questions requiring further study [1]. Dissecting the biophysical coupling between force-regulated TCR–pMHC bonding, TCR–CD3 coupling, and CD3–membrane association would provide the key evidence to integrate force into the TCR triggering models. The cooperation between TCR and coreceptor CD8/CD4 that manifests a dynamic catch with pMHC also requires more detailed characterization of the intracellular molecular assembly and its dynamic regulation [17]. Moreover, T cell activation is not determined by TCR signaling alone. We expect to see more studies on the mechanobiology of costimulatory/co-inhibitory receptors, their spatial regulation, and signaling dynamics during T-cell activation. Finally, these studies would have great potential to empower the development of advanced T cell therapy by engineering on-demand signaling and functions of (artificial) antigen receptors as well as co-stimulatory/co-inhibitory receptors. One immediate implication for conventional and Chimeric Antigen Receptor (CAR) T cell therapy is to integrate mechanical force into the screening and design of the antigen receptors for more efficient recognition. Also, design of bispecific antibodies that crosslink antigen receptor and target cells may further increase the valency for clustering-based signaling amplification [63].

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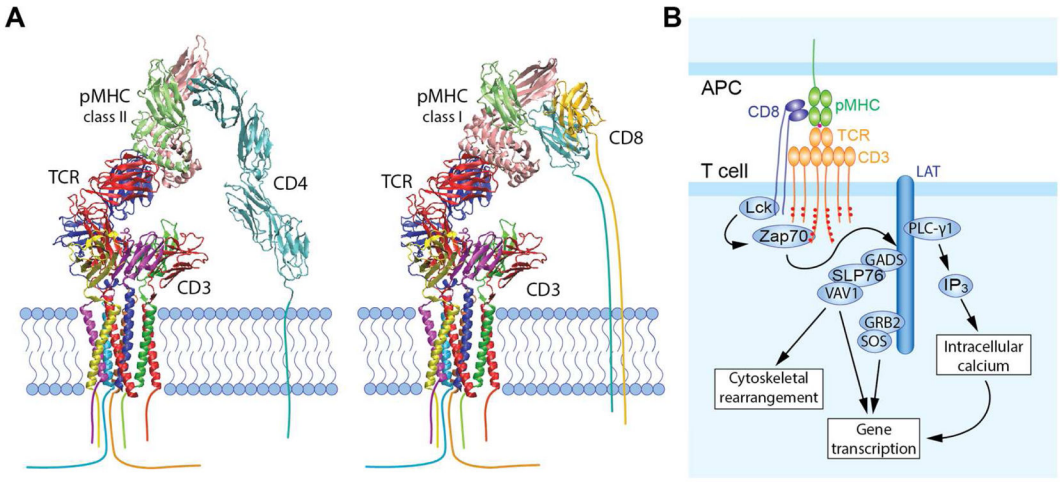

Figure 1.

The TCR–CD3–coreceptor antigen recognition apparatus and its signaling pathway. **A**, Modeled structure of TCR-CD3 in complex with class II pMHC and CD4 (left) or with class I pMHC and CD8 (right). **B**, Binding of TCR and CD8 (or CD4) triggers the proximal signaling that involves phosphorylation of CD3 ITAMs by Lck, ITAM binding and activation of Zap70, and phosphorylation of LAT. Recruitment of multiple adaptor and signaling molecules to LAT and their activation further propagate and direct downstream signaling for various cellular functions.

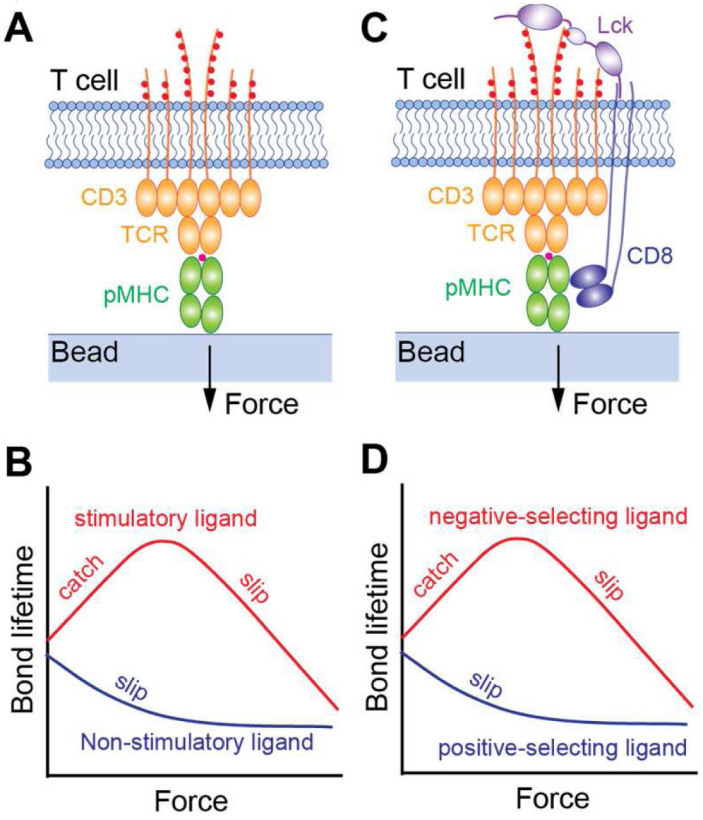

Figure 2.

Association of TCR–pMHC and TCR–pMHC–CD8 bond patterns with functional outcomes. **A-B**, TCR forms intrinsic catch bond with agonist (stimulatory) ligands and slip bond with non-agonist (non-stimulatory) ligands. **C-D**, TCR and CD8 form Lck- and CD3-dependent dynamic catch with negative selection ligands and slip bond with positive selection ligands.

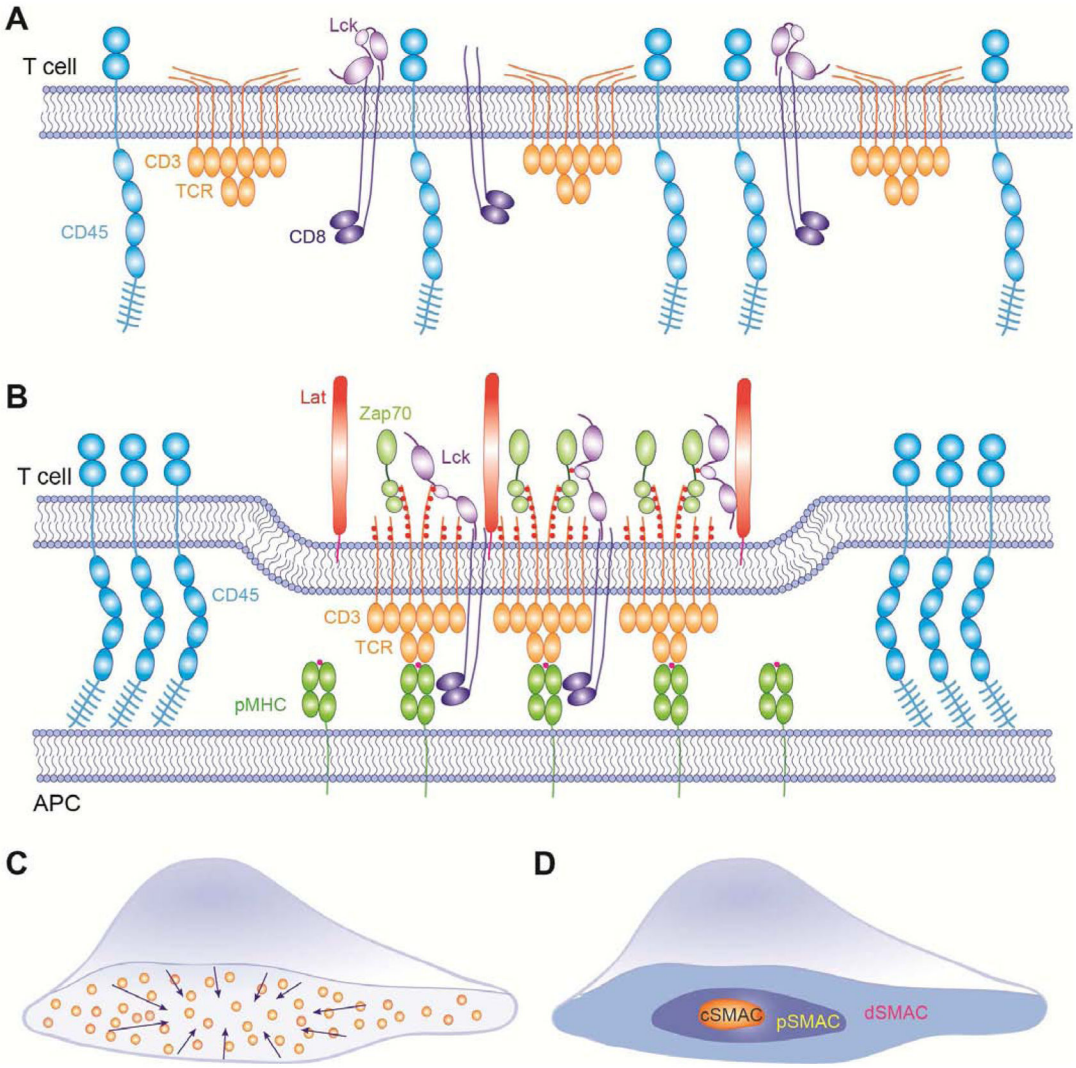

Figure 3.

Spatial regulation of T-cell activation. **A**, On resting T cell surface, phosphorylation of TCR-CD3 is prevented by the membrane-association of CD3, the auto-inhibition of Lck kinase activity, and far greater number of phosphatase CD45 than Lck in TCR-CD3 proximity. **B**, TCR-pMHC binding leads to exclusion of CD45, phosphorylation of CD3 ITAMs, and docking of Zap70 and Lck. This further enhances the recruitment of CD8 and Lat. **C**, The segregated TCR and the local assembly of multimolecular signaling complex merge into bigger microclusters and move towards the center of the immunological synapse. **D**, Large scale molecular reorganization results in the bull's eye pattern of cSMAC, pSMAC, and dSMAC.