Published in final edited form as:

Immunol Rev. 2009 November ; 232(1): 7–21. doi:10.1111/j.1600-065X.2009.00843.x.

Organization of proximal signal initiation at the TCR:CD3 complex

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Summary

The series of events leading to T cell activation following antigen recognition has been extensively investigated. Although the exact mechanisms of ligand binding and transmission of this extracellular interaction into a productive intracellular signaling sequence remains incomplete, it has been known for many years that the immunoreceptor tyrosine activation motifs (ITAMs) of the T cell receptor (TCR):CD3 complex are required for initiation of this signaling cascade due to the recruitment and activation of multiple protein tyrosine kinases, signaling intermediates and adapter molecules. It however remains unclear why the TCR:CD3 complex requires 10 ITAMs, whilst many other ITAM-containing immune receptors, such as Fc receptors (FcR) and the B cell receptor (BCR), contain far fewer ITAMs. We have recently demonstrated that various parameters of T cell development and activation are influenced by the number, as well as location and type, of ITAM within the TCR:CD3 complex and hence propose that the TCR is capable of 'scalable signaling' that facilitates the initiation and orchestration of diverse T cell functions (1). While many of the underlying mechanisms remain hypothetical, this review intends to amalgamate what we have learned from conventional biochemical analyses, regarding initiation and diversification of T cell signaling, with more recent evidence from molecular and fluorescent microscopic analyses to propose a broader purpose for the TCR:CD3 ITAMs. Rather than simply signal initiation, individual ITAMs may also be responsible for the differential recruitment of signaling and regulatory molecules which ultimately affects T cell development, activation and differentiation.

Keywords

T-cell receptor; CD3 complex; T-cell signaling; immunoreceptor tyrosine activation motif

Introduction

Developing thymocytes and mature T cells sample the spectrum of antigenic peptides displayed on antigen-presenting cells (APCs) or cells of various tissues, via a complex comprised of heterodimeric T-cell receptor (TCR) α and β chains (2) in combination with four CD3 subunits, denoted ϵ , γ , δ and ζ , which associate with TCR $\alpha\beta$ as three dimers ($\epsilon\gamma$, $\epsilon\delta$, $\zeta\zeta$) (3,4). This TCR:CD3 complex therefore serves as the conduit for initiation of essentially all adaptive immune responses and, as such, has been the focus of intensive research since the TCR was first identified (5–7). One of the lingering questions remains the necessity for such a complicated receptor complex for the initiation of T-cell activation. While many immune receptors contain cytoplasmic regions with immunoreceptor tyrosine-based activation motifs (ITAMs) (YXXL/I-X₆₋₈-YXXL/I), the TCR:CD3 complex is unique

in that it contains a total of 10 such motifs which are contributed by the various CD3 subunits. CD3 ϵ , CD3 γ and CD3 δ each contribute a single ITAM while the CD3 ζ homodimer contains 3 ITAMs. The three CD3 dimers [$\epsilon\gamma$, $\epsilon\delta$, $\zeta\zeta$] that assemble with the TCR thus contribute 10 ITAMs. Phosphorylation of the tandem tyrosine residues upon TCR ligation creates paired docking sites for proteins that contain Src homology 2 (SH2) domains such as ζ chain-associated protein of 70 kDa (ZAP-70), thereby initiating a signaling cascade which leads to T-cell activation and differentiation (discussed in detail below). In a similar cascade of events, B cells are activated via the B-cell receptor (8), while activation signals are also perceived and transmitted through ITAM-containing proteins in myeloid and other hematopoietic cells. However, a comparative analysis of these receptors would suggest that the TCR:CD3 complex contains an overabundance of ITAMs in comparison with B cells and other lymphoid effectors whose receptors contain only 1 or 2 ITAMs. It has been suggested that a full complement of ITAMs is necessary for T-cell development and effector function, as discrete parameters of activation including phosphorylation of signaling proteins (9,10) or induction of cell death (11) may be differentially affected by the various ITAMs of the CD3 subunits (12,13). Thus, while ITAM multiplicity may impart partial redundancy during T-cell activation, it remains possible that the role of individual ITAMs may be to differentially recruit signaling molecules, thereby engaging separate pathways of activation. Since the ITAMs of the CD3 complex are the key initiators of T-cell activation, this review highlights the importance of ITAMs in T-cell activation and places our understanding into a framework of what we know (reviewed in 14) and what we postulate about TCR-induced signaling.

TCR ligation: porthole to the antigenic world

Twenty-five years ago, the works of several laboratories provided the first conclusive evidence regarding the nature of the receptor responsible for encoding T-cell reactivity (5–7). Since these early pioneering studies, a quarter century of research has exponentially expanded our biochemical and morphological understanding of the TCR:CD3 complex. More recent structural evidence continues to advance our understanding by suggesting an additional level of TCR control (15) above that simply suggested by the long standing dogma that TCR aggregation was required for signal propagation (16). The mechanism (s) underlying translation of ligand recognition by the TCR, into an outside-in signal culminating in T-cell activation, is an area of intense investigation along parallel, non-exclusive lines. Research focused on conformational changes in the TCR upon ligand recognition has resulted in the consensus that much of the flexibility observed upon TCR-peptide/major histocompatibility complex (pMHC) binding occurs in the CDR3 regions of the TCR chains (reviewed in 17). Additionally, a recent study suggests that agonist but not antagonist binding yields a conformational change in the A-B loop of the α -chain constant domain (18) and may reflect the earliest structural change induced upon antigen recognition.

Since there are estimated to be as few as several hundred T cells capable of recognizing any single antigen and thousands of antigens presented at any one time on the surface of an APC, intensive investigation has attempted to define how many TCR engagements are required by a T cell to initiate a response leading to activation, proliferation, and differentiation. An elegant series of experiments demonstrated that interaction of the TCR with a single pMHC is capable of inducing a complex between T cell and APC with 95% occurrence, leading to an increase in intracellular calcium (19). Interestingly, the calcium response was observed to plateau upon stimulation with 20–30 agonist peptides, while the formation of a stable immunological synapse (discussed below) required 10 such interactions. Coreceptor engagement became necessary for amplification of the response if APC were loaded with <25 agonist peptides, suggesting that the TCR is inherently poised to respond to very low frequencies of agonist peptide. The complete contribution of

endogenous pMHC recognition by the TCR to initiation of T-cell signaling is as yet unclear; however, previous work (19) clearly demonstrated an aggregation of MHC molecules within the central region of the immunological synapse that were not presenting agonist peptide. Whether or not the mode of action can be attributed to serial TCR triggering and amplification of signal (20) or due to the specific interaction of TCR-agonist pMHC complexes as a result of enhanced Lck activity induced by endogenous pMHC recognition (21) is uncertain (reviewed in 2).

Are CD3 subunits structurally active and/or relevant?

While it has long been recognized that coordinate expression of both CD3 subunits and the heterodimeric TCR is obligatory for surface expression of a functional complex (22), how TCR-mediated pMHC recognition is transmitted to the CD3 complex leading to activation is unresolved. Given the close association of the TCR and CD3 molecules, a conformational change in the TCR may influence the positioning of the CD3 subunits within the complex (23) and alter accessibility of cytoplasmic CD3 binding motifs to the actions of protein tyrosine kinases (PTKs) or adapter molecules.

A contribution of a CD3 conformational change to the induction of T-cell activation is suggested by the long standing observation that the proline-rich sequence (PRS) of CD3 ϵ recruits the adapter molecule Nck and that this even occurs prior to ITAM phosphorylation (24). Elimination of the CD3 ϵ PRS suggested its involvement with the adapter molecule SLAP, leading to the down regulation of the TCR and enhanced sensitivity to antigens in developing DP thymocytes (25). However, a more recent study suggests that the capacity of CD3 ϵ to bind Nck is not generally required for T-cell development or activation (26), although it may serve to amplify TCR signaling in response to weak stimuli (27) or to regulate ITAM phosphorylation and TCR internalization (28). More convincing evidence of the requirement for conformational changes in CD3 to facilitate TCR signaling has been demonstrated in an elegant study (15) wherein key tyrosine residues of the CD3 ϵ ITAMs were determined to reside within the hydrophobic core of the plasma membrane as a result of the interaction of membrane-proximal basic residues and which become accessible to cytoplasmic PTKs upon TCR ligation, complementing previously reported findings (29). Several studies also support the notion that not only are the intracellular regions of the CD3 subunits important for signal transmission but that the interaction of the extracellular domains with the TCR are important for T-cell activity (23). This has been elegantly shown for the CD3 δ subunit, wherein a tail-less molecule is sufficient to rescue thymocyte development and ERK activation (30). Likewise, a mutation of the TCR that modifies its association with the CD3 δ subunit drew similar conclusions (31). Interestingly, the latter study suggested that high affinity TCR ligands induced a transient activation of ERK, while low-affinity ligand stimulation yielded sustained ERK activity, implying other regulatory mechanisms within the TCR signaling complex which may influence this component of the TCR-induced signal cascade.

Models of TCR:CD3 complex triggering

Considering the extensive research conducted in this area and an as yet elusive understanding of the precise structural changes required for signal transmission across the plasma membrane, several models of TCR triggering have been proposed. These include the prediction that the external force of TCR binding creates a piston-like movement, or torque (32), resulting in movement of the entire TCR:CD3 complex deeper into the plasma membrane and exposing cytoplasmic motifs leading to activation, or that the coupling of agonist peptide recognition by the TCR, followed by CD4-mediated binding to endogenous pMHC, allows amplification of signal via activation of Lck. Similarly, it has also been

suggested that the dynamic association of T cells with APCs presenting cognate pMHC may be an additional initiating factor for TCR triggering (33). Briefly, these authors propose that as a consequence of cytoskeletal remodeling and the affinity of binding between TCR:pMHC complexes, membrane dynamics impart a physical force upon the TCR complex. This force is transmitted into a conformational change within the complex, thereby facilitating accessibility of the CD3 subunits to the actions of PTKs such as Lck. It has been hypothesized that key polar interactions between the TCR complex and the CD3 dimers within the plasma membrane, may communicate transmission of this external conformational change (3). Further, the association of CD3 ϵ and ζ chains with a lipid environment precludes their phosphorylation (15,34). Thus, it would seem that remodeling of the plasma membrane and reorganization of the TCR:CD3 complex with respect to the lipid environment may be required for T-cell activation.

A more recent addition to the family of proposed TCR triggering models, the kinetic segregation model (35), proposes that upon contact with APC (or presumably a lipid bilayer loaded with MHC and adhesion molecules including CD2 (36), proteins segregate within the membrane partially due to size differences and steric restraints. In such a manner, TCR triggering induces a signaling event (discussed below) which is amplified and maintained due to the exclusion of large, bulkier protein tyrosine phosphatases (PTPs) including CD45. Since phosphorylation of tyrosines within the CD3 subunits ITAM sequences is promoted by PTKs and maintained due to phosphatase exclusion, signaling is sufficient to drive activation. This model is certainly closely aligned with one which proposes that individual TCRs are retained in an inactive conformation within positively charged areas of the cell membrane, due to constraint provided by the actin cytoskeleton (37). Conformational change allows tyrosine residues buried within the plasma membrane (15) to become accessible to tyrosine kinases. Coupling of TCR phosphorylation and signaling to the actin cytoskeleton redistributes the TCR to a more ordered area of the plasma membrane which excludes larger molecules and phosphatases such as CD45, prolonging TCR phosphorylation as a result of enrichment in these areas for Src family kinases.

Most of the models mentioned above are not mutually exclusive and are all likely partially relevant to the chain of events leading to effective TCR:CD3 complex activation. How does mutation of ITAM sequences influence early events in TCR:CD3 complex activation? It is conceivable that the inability to phosphorylate particular ITAMs within the CD3 subunits may affect stability of the triggered complex or influence the movement of the various CD3 dimers. It also remains possible that varying the number of wildtype and hence 'activated' ITAMs upsets the kinetics and capacity of the triggered TCR:CD3 complexes to be sequestered from the effects of phosphatases. Thus, while the outcome of TCR activation in the absence of a full complement of wildtype ITAMs is distinctly impaired in some capacities and not others (1), the mechanism (s) responsible for differential patterns of activation in these retrogenic mice are currently unknown and is (are) a matter for investigation.

One possible research question is to understand whether the positioning of the functional ITAM subunits is integral to the activation of downstream signaling. TCR:CD3 complex assembly is postulated to be in part reliant upon amino acid residues within the transmembrane regions of the TCR α and β chains, and the various CD3 subunits (23). Thus, the TCR α chain interacts via distinct residues with both the CD3 $\delta\epsilon$ and CD3 $\zeta\zeta$ subunits, while the TCR β chain exhibits association with the CD3 $\gamma\epsilon$ heterodimer. While the precise nature of the extracellular contacts necessary for complex assembly are less certain, it has been proposed that the CD3 heterodimers are opposed with respect to their interaction with the TCR (38). Thus, while the exact mechanisms of TCR triggering and conformational changes discussed above are still uncertain, structural evidence may suggest that the

organization of the TCR:CD3 complex may predetermine the sequence of events, including ITAM phosphorylation and availability following antigen recognition. The relative importance of ITAM location within this three dimensional structure to the outcome of TCR signaling is an exciting area of future research.

Immunological synapse formation and initiation of T-cell signaling

The molecular structure of the immunological synapse as a platform for TCR:CD3 signaling was formally proposed following the groundbreaking use of planar lipid bilayers to analyze T-cell activation (36). However, it was earlier work that initially described an aggregation of TCR:MHC clusters, present in fixed T and B-cell conjugates, which was termed central supramolecular activation cluster (cSMAC) (39). This molecular platform was presumed to facilitate T-cell activation, in part due to its apparent recruitment of protein kinase C θ (PKC θ) and the PTK Lck. In contrast, the peripheral supramolecular activation cluster (pSMAC) was found to be enriched for adhesion molecules, and in particular demonstrated the interaction of intercellular adhesion molecule-1 (ICAM-1) with leukocyte function-associated antigen-1 (LFA-1). These findings (39) reinforced the previously proposed segregation model (40) and confirmed suggestions from a previous work that demonstrated that accumulation of CD4 and polarization of the cytoskeleton resulted in the full activation and differentiation of T cells (41). The concept that T cells formed a stable signaling platform was subsequently demonstrated (42) and revealed that adhesion mediated by LFA-1 and CD2 resulted in polarization of the T-cell cytoskeleton towards the point of contact with APC or planar bilayer, and in so doing, formed a stable synapse between the stimulatory surface and responding T cell. Formation of this macromolecular complex is necessary for propagation of a sustained signaling interaction. Subsequent investigations sought to more precisely determine the sequence of molecular events underlying TCR:CD3 signaling. A key series of experiments suggested that the immunological synapse formation was initiated in microclusters (43), which are subsequently transported via both F-actin and myosin II-dependent mechanisms (44) leading to formation of the mature synapse previously reported (39). Further, this work demonstrated that the initiation of TCR signaling occurred prior to the construction of the cSMAC, as evidenced by increased intracellular Ca^{++} in response to microcluster formation (43). The importance of microclusters in signal initiation was further highlighted by data that confirmed their involvement in the activation of Lck, and in contrast with earlier work (39), suggested that the cSMAC was an area of TCR signal termination (45). The results of this work clearly supported the notion that TCR:CD3 signaling occurred in microclusters, as evidenced by phosphotyrosine detection, while the cSMAC is largely devoid of activated TCRs but is rather associated with their downregulation suggested to occur via a CD2AP-dependent mechanism (45,46). Subsequent work highlighted the importance of signaling in TCR microclusters (47) through their recruitment of key signaling intermediates Lck, ZAP-70, and SH2 domain-containing protein of 76 kDa (SLP-76) (48,49) and the induction of calcium-dependent signaling (47) prior to the coalescence of these microclusters into the mature cSMAC.

Evolving views of synapse morphology and function

It has been recently suggested (37) that the role of the cSMAC may be twofold, representing an area enriched for TCR which is to be internalized and degraded (50), while concomitantly forming a signaling complex comprised of F-actin, CD28, and the PKC θ originally observed (39). Further, the definition of immunological synapse should be modified to incorporate the observation that T cells may exhibit differing rates of synapse formation, breakage, and reformation, depending upon the relative contributions of PKC θ and Wiskott-Aldrich syndrome protein (WASp) to this dynamic process (51;52), and hence the term

'immunological kinapse' has been proposed (53). Thus, our understanding of the role played by membrane organization of signaling moieties in the initiation of T-cell responses continues to evolve from that originally described (39), to that of a more dynamic entity. Certainly, it is now recognized that immunological synapse formation may ultimately result in a variety of morphological structures with varied signaling and functional outcomes (reviewed in 54). While the term 'immunological synapse' may predominantly invoke images of a bull's eye comprised of the cSMAC, pSMAC, and distal supramolecular activation cluster (dSMAC), it has become apparent that this classical morphology is not exclusive. Rather, developing thymocytes form a multifocal synapse during negative selection (55), which is supported by recent data suggesting that T-cell maturation and positive selection proceeds in the absence of a synaptic contact with thymic epithelium (56). Further, it has been recently shown that T-helper 1 (Th1) and Th2 effector cells form distinctly different synapses, wherein the morphology of a Th1 cell synapse is a classical bull's eye, while that of a Th2 cell is multifocal (57). However, the ultimate morphological appearance of the immunological synapse is not entirely T-cell dependent, since dendritic cell-T cell couples form multifocal synapses (58) in contrast to those observed during B-T cell interactions, or those analyzed on planar lipid bilayers.

Since the formation of microclusters and their coalescence into a mature cSMAC is reliant upon the actin cytoskeleton (47) and myosin motor proteins (44), while continued breakage and reformation are reliant upon PKC θ and cytoskeletal adapter proteins, respectively, it remains entirely conceivable that the formation of a mature synapse may be dysregulated in CD3 ITAM mutant mice wherein a reduced capacity to phosphorylate a full complement of ITAMs may not fully engage a complete T-cell activation program (see discussed in detail below). Thus, deficiencies in synapse formation (or deformation) may initiate signaling defects in developing thymocytes leading to a lack of negative selection, while manifesting as a partial activator of mature T cells in the periphery leading to cytokine production in the absence of a proliferative response (1). While it remains possible that mutant T cells containing only 1 or 2 functional ITAMs may also exhibit deficiencies in microcluster formation, our data thus far suggest that while these cells are profoundly deficient in their proliferative capacity, TCR stimulation does yield a similar cytokine profile in both wildtype and ITAM-mutant mice. Thus, we would expect that at least minimal TCR:CD3 signaling and thus initial microcluster formation is preserved in these mice, and we have evidence to suggest that this portion of the TCR-induced signaling cascade is intact in mice carrying only four or two functional ITAMs (C Guy and D Vignali, unpublished observations). These data are consistent with a recent study where microcluster formation was shown to be independent of Src kinase activity (48).

CD3 subunit ITAMs as initiators of T-cell signaling: are all subunits equal?

Early work that endeavored to discover how the TCR complex transmitted activating signals following ligand binding determined that tyrosine phosphorylation of the complex was a prerequisite for PKC activation and elevations in intracellular Ca⁺⁺ (59). The importance of the CD3 ITAMs was realized when several groups created chimeric proteins with cytoplasmic tails derived from CD3 ζ (60) or CD3 ϵ (10), which imparted TCR:CD3 signaling capacity. A subsequent study provided the first evidence that phosphorylated CD3 ITAMs provided a docking site for ZAP-70 (61). The existence of two tandem SH2 domains within the ZAP-70 molecule likely precludes its binding to singly phosphorylated ITAMs, since both domains have been shown to be tightly apposed and closely associated with the completely phosphorylated ITAM (62), yielding binding affinities reported in the range of 20 nM (63). The importance of a doubly phosphorylated ITAM sequence for T-cell activation has been suggested by others as a key regulatory feature responsible for the recruitment of ZAP-70, since partial agonists, which result in the incomplete

phosphorylation of the CD3 ζ chain, fail to activate and associate with ZAP-70 (64). Characterization of p21 as containing monophosphorylated ITAMs and that of p23 as containing diphosphorylated ITAMs, suggested a negative role for p21 in T-cell activation upon TCR ligation (65), possibly due to the binding of negative regulators such as SHP-1 (66). One study reported that the phosphorylation of the CD3 ζ chain occurs in a stepwise manner, initiated by phosphorylation of the C-terminal ITAM tyrosines leading to formation of the intermediate p21 isoform, and ultimately to the fully phosphorylated ζ chain, or p23 isoform (67). Interestingly, TCR signals can proceed independently of ζ chain phosphorylation, suggesting that the CD3 ϵ , γ , and δ subunits can impart sufficient signals to induce T-cell activation as measured by interleukin-2 (IL-2) production, consistent with our recent findings (1).

Several papers published in the mid-1990s suggested differential affinities of the various phosphorylated ITAMs for signaling intermediates and adapter proteins. Two reports (68,69) suggested the hierarchical affinity of ZAP-70 for the phosphorylated ITAMs of the CD3 ζ subunit, wherein the membrane proximal ITAM exhibited the highest binding affinity and the membrane distal ITAM was determined to be of lowest affinity. The latter work also suggested that other kinases such as Shc and phosphoinositol 3-kinase (PI3K) as well as adapter molecules like Grb2 and GAP exhibited differential affinities for di- and monophosphorylated CD3 ζ subunits. For example, Grb2 does not associate with the membrane distal ITAM, Shc is not recruited to the middle ITAM subunit, while PI3K is capable of binding both di- and monophosphorylated subunits irrespective of location. A subsequent study (70) determined that the binding affinity of the CD3 ϵ ITAM was intermediately located between the binding constants observed for the middle and membrane distal ITAMs of the CD3 ζ chain. *In vitro* assays demonstrated varying binding affinities of the various phosphorylated ITAM peptide sequences to ZAP-70, suggesting that flanking residues, or those which intervene between the paired tyrosines within the ITAM sequence, may differentially contribute to the initiation of TCR signaling (68), while a similar comparison of binding affinities extended previous findings by inclusion of the CD3 γ and CD3 δ ITAM sequences as well (71).

A cautionary note to the *in vivo* relevance of the above findings has been suggested by a report that suggests that while recruitment of ZAP-70 to the membrane is sufficient for its activation (72). Proper configuration of the activated molecule is necessary for interaction with downstream effectors, leading to complete T-cell activation. Thus, the results of the aforementioned *in vitro* analyses using synthesized peptides may not accurately reflect the interactions of the intact proteins as they assemble within the scaffold of the TCR signalosome. Further, disruption of one or more functional ITAMs within the context of the entire signaling complex may alter the kinetics of activation, particularly with respect to the CD3 ζ chain considering the results of *in vitro* assays suggesting stepwise activation of the fully phosphorylated p23 isoform. Our previous data suggests that Y-F substitutions in various ITAM subunits yields subtle differences in developmental parameters, while exhibiting a more quantitative effect on other aspects of T cell activation (1). Thus, it remains possible that an ordered series of activation or differential recruitment of some signaling molecules to the TCR:CD3 complex is necessary for some but not all parameters of T-cell activity. To further address this issue, retroviral mediated reconstitution of a signaling complex comprised of 10 functional ITAMs, but of a single variety (i.e. CD3 ϵ , γ , δ , ζ a, ζ b or ζ c), may shed valuable light on the relative importance of each subunit and its capacity to engage ZAP-70 or other key molecules for the successful initiation of T-cell activation during thymic development or as a result of stimulation of mature T cells.

TCR:CD3 signaling beyond ITAM initiation

It has been proposed that one of the reasons for ITAM multiplicity within the TCR:CD3 complex is to facilitate signal amplification by increasing the local concentration of signaling intermediates and effector molecules (12). It has been known from an early point since the investigation of TCR signaling that T-cell activation requires PTK function (73), while the tyrosine kinase Lck was previously known to associate with either the CD4 or CD8 coreceptors (74,75), which have been shown to decrease the TCR triggering threshold required for T-cell activation (19). As previously mentioned, doubly phosphorylated ITAMs provide tandem SH2 docking sites for ZAP-70 recruitment and activation, yielding a molecule which can be phosphorylated by Lck on 3 tyrosine residues (reviewed in 76). Early studies demonstrated that a Y292F mutation resulted in enhanced NFAT activity using a B-cell receptor system (77), while the converse observation that the E3 ligase c-Cbl promotes internalization and degradation of the complex in response to Y292 phosphorylation (78,79) together suggest a regulatory role for this activating tyrosine residue (further discussed below). Phosphorylation of tyrosine 315 has been suggested to link TCR:CD3 signaling to the actin cytoskeleton, since Y315F is impaired in the recruitment of Vav to phosphorylated B cell receptors *in vitro* (80); however, this mechanism seems dispensable *in vivo* (81). Additionally, a Y315F mutation demonstrated that the adapter protein Crkl is similarly impaired in its recruitment to the TCR:CD3 complex suggesting defective formation of a complex including WASp and WIP, and reorganization of the cytoskeleton (82). Further, a Y315F mutation yields diminished PLC γ and ERK phosphorylation with concomitant decreases in Ca⁺⁺ mobilization (81,83). Early studies suggested that TCR (or CD3) activation resulted in elevated intracellular Ca⁺⁺ levels, in a manner which could be recapitulated by stimulation with phorbol esters or calcium ionophores (84), leading to the widely accepted notion that PLC γ was a key initiator of T-cell responses due to its ability to produce the intermediaries IP3 and DAG. In this regard, phosphorylation of the Y319 residue of ZAP-70 may represent a more important regulator of downstream T-cell activation, as this site has been shown to bind PLC γ (85) thereby strongly impacting Ca⁺⁺ mobilization and thymocyte development. Recent investigations suggest that a measure of caution should be included in the interpretation of the above data wherein YYFF mutations may not simply disrupt ZAP-70 function as a result of their decreased capacity to act as a signaling scaffold, but rather that phenylalanine substitutions imparted stability to the inactive ZAP-70 structure. The authors therefore propose that key tyrosine residues are not a necessity for signal propagation directly, but rather contribute to the determination of catalytic activity via an autoinhibitory switch (reviewed in 76).

Importance of the TCR signalosome

One of the key ZAP-70 substrates responsible for TCR signal transmission is linker for activation of T cells (LAT) (86), a prerequisite molecule not only for T-cell activation but also for T-cell development (87). LAT is a transmembrane protein which is comprised of an extended cytoplasmic tail and a short extracellular domain. The existence of multiple tyrosine residues within the cytoplasmic tail of LAT suggested that phosphorylation of these residues by ZAP-70 would facilitate the adapter function of LAT through its capacity to concomitantly recruit multiple SH2 domain-containing signaling proteins (reviewed in 88). Clustering of LAT within the immunological synapse is inhibited by the Src kinase inhibitor PP2 (89), while it has been shown that its subsequent partitioning into glycolipid-enriched microdomains mediates T-cell activation by PLC γ and the guanine nucleotide exchange factor Ras (90,91). Subsequent studies have placed LAT at a key junction of T-cell activation due to its ability to recruit directly or indirectly several key signaling intermediates including SLP-76, Grb2, and other components of the TCR:CD3 signalosome such as Vav, Cbl, and PI3K. SLP-76 is without question a key component of this molecular

superstructure, since loss of SLP-76 represents a lethal hit to T-cell activation (92). Thus SLP-76 along with LAT forms the core signalosome complex for the diversification of the TCR:CD3 signal, initiated by ITAM phosphorylation, which initiates several key signaling pathways. The importance of both LAT and SLP-76 has been determined previously, as T cells deficient in both signaling molecules are incapable of initiating intracellular calcium-flux or downstream expression of IL-2 in response to TCR engagement, although CD3 ζ and ZAP-70 phosphorylation are unperturbed (93). SLP-76 has been conclusively placed downstream of LAT activation, wherein it mediates the formation of a trimolecular complex comprised of SLP-76, Vav, and Nck. This in turn recruits and activates the Rho GTPases, thereby coupling TCR:CD3 signals to cytoskeletal remodeling (94), as well as initiating signaling through the Ras/MAPK and PKC signaling pathways (reviewed in 95).

Complementing TCR signals through Costimulation

Although TCR stimulation is a prerequisite for T-cell activation, it is well recognized that engagement of costimulatory molecules, such as CD28, is necessary for full T-cell activation and differentiation. CD28 has been shown to influence multiple parameters of TCR-induced signaling, including those which are both proximal as well as distal to TCR crosslinking (96). Interestingly, it has been suggested that CD28 costimulatory signaling in the absence of a TCR signal is sufficient to drive cytokine production as a result of phosphorylated SLP-76 and subsequent recruitment of Vav (97). These data may be relevant to our recent findings, wherein ITAM mutant retrogenic T cells exhibited marked proliferative defects but unimpaired cytokine secretion (1). Specifically, a decreased TCR:CD3 signal imparted by deficiencies in ITAM phosphorylation and recruitment of signaling adapters such as Vav may be insufficient to drive proliferative responses; however, the provision of CD28 stimulation in these cultures, either through the use of antigen-pulsed APC or soluble CD28 antibodies, may be sufficient to induce cytokine production in the absence of a fully activated TCR:CD3 signaling program.

Analysis of TCR:CD3 signaling capacity coincident with CD28 stimulation is also confounded due to CD28-mediated localization and activation of PKC θ to the area of the immunological synapse, in a manner which is Lck dependent but ZAP-70 independent (98). Subsequent analyses confirmed these findings and further suggested that PKC θ activation during T-cell stimulation is predominantly independent of TCR:CD3 signaling, as CD28 and PKC θ were found to be enriched in the cSMAC (39,99). Deficits observed in PKC θ knockout mice can be largely mitigated upon addition of exogenous IL-2, suggesting that the predominant role for PKC θ is transcription of IL-2 mediated by activation of key transcription factors activator protein 1 (AP1) and nuclear factor κ B (NF κ B) (100). Congruent with our understanding that PKC θ activation via costimulatory molecules is the predominant pathway leading to IL-2 production, T cells derived from ITAM mutant mice did not exhibit significant defects in secretion of cytokines, including IL-2 (1). Thus, we may speculate that deficiencies in ITAM phosphorylation are not meaningfully detrimental to the downstream activation of transcription factors involved in cytokine gene transcription, in part due to the role played by CD28 under the stimulatory conditions performed (1). However, an exhaustive analysis to determine the contribution of ITAM signal strength in the absence of costimulation has not been performed. Furthermore, whether a temporal dysregulation of cytokine expression occurs during the early priming of ITAM mutant T cells has not been fully assessed.

Strength of ITAM signaling and T-cell development

It has been known for more than 15 years that mice deficient in the CD3 ζ chain exhibit impaired thymocyte development and a decreased number of peripheral T cells (101), which

have been determined to display enhanced autoreactivity (102). These observations suggested that the number of ITAMs can modify strength of signal imparted by selecting ligands leading to defective negative selection during thymic development, which has been recently supported by our reported findings (1). Experiments using transgenic mice deficient in other CD3 components (103–105) resulted in markedly impaired T-cell development and precluded evaluation of how phosphorylation of specific CD3 subunit ITAMs contributed to TCR signaling during thymocyte selection.

It has been known for some time that ZAP-70 is partially required for both positive and negative selection (106), while a double deficiency in Syk and ZAP-70 results in a complete block in T-cell development (107). In the latter, immature T cells are arrested at the double negative 3 (DN3) stage, suggesting a requirement for both kinases in mediating pre-TCR signaling. Syk is required for TCR β signaling, leading to the sequential upregulation and activity of ZAP-70, which is necessary for progression through the DN4 and single positive (SP) stages of development (108). Thus, a recently proposed model suggests that Syk is preferentially used during early T-cell development, while later stages are more dependent on ZAP-70 (76). This may be due to its association with either CD4 or CD8 coreceptors and/or due to its capacity to activate p38 MAPK.

Decreased TCR signal strength imparted by mutation of the CD3 ζ ITAMs resulted in impaired thymocyte development but not homeostatic regulation, when moderate affinity TCRs were utilized (109). In contrast, high affinity TCR interactions were sufficient to overcome decreased CD3 signal strength. A similar study determined that expression of a CD3 ϵ mutant with a Y-F substitution within the ITAM sequence was sufficient to restore surface TCR expression on developing thymocytes and that inability to signal through phosphorylation of the CD3 ϵ subunit did not affect T-cell development detrimentally (110). Surprisingly, the proliferative and cytokine response of peripheral T cells was deficient in CD3 ϵ ITAM mutant mice (i.e. retaining a total of 8 functional ITAMs within CD3 δ , γ , and ζ). While the latter observation is partially consistent with our recently reported findings (1), it cannot be excluded that the TCR model system used and hence TCR affinity may also contribute to the relative capacity of T cells to respond to stimulatory ligands in the absence of a full complement of ITAMs. In related work, it has been shown that under normal T-cell stimulatory circumstances, the complete phosphorylation of the CD3 ζ chain occurs only if the carboxy-terminal tyrosine residue of the membrane proximal sequence is intact, suggesting that ITAM phosphorylation follows an ordered program of activation as previously suggested (111). T-cell stimulation with altered peptide ligands (APLs) of varying affinities yielded partially phosphorylated ζ subunits, while the composition of which tyrosines were phosphorylated differed with the strength of stimulus. Although the nature of the selecting ligands utilized during thymic development in our recently described ITAM mutants are unknown, it remains possible that the status of ζ chain phosphorylation was an important determinant in the development of autoreactive T cells, which was due to a reduced signal and lack of negative selection. Specifically, ITAM mutant mice which had a total of 6 intact ITAMs developed autoimmunity in 2 of the 4 possible combinations (1). Intriguingly, if the ζ chain contained an intact second ITAM (i.e. the ζ _b subunit), the mice did not develop autoimmunity, while silencing of this ITAM in combination with either the ζ _a or ζ _c ITAM was sufficient to alter the development and activation of peripheral T cells capable of recognizing self-antigen and initiation of autoimmunity. A recent study suggested that the development of HY antigen-specific T cells was impaired in CD3 ζ transgenic mice lacking all or a combination of the ζ ITAMs (112). Together, the results of this study and of our recently reported data suggest that decreased signal strength imparted by a decrease in functional ITAMs can either negatively or positively affect T-cell development depending on the selecting ligand and the context of presentation. Interestingly, the activation of peripheral HY-specific CD8⁺ T cells was largely unimpaired in CD3 ζ transgenic mice (112),

while CD4⁺ T cells derived from CD3 ITAM-mutant mice exhibited a profound defect in proliferation (1). It is possible, however, that decreased signal strength is of greater detriment to the activation of CD4⁺ cells compared with CD8⁺ effectors, since it is known that CD8⁺ cells require a lower threshold of activation (113), possibly due in part to the more readily accessible peptides presented by MHC class I in comparison to those buried deep within the pocket of MHC class II molecules (reviewed in 17). These results therefore suggest that an investigation of ITAM function in CD4⁺ T cells of varying TCR affinity would be worthwhile, providing evidence to support or refute the possibility that affinity and ligand engagement provides an additional level of regulation to that of CD3 signal strength, on the outcome of TCR-induced signaling.

TCR:CD3 signaling and T-cell differentiation

One of the functions of the immunological synapse is that by co-recruitment of adhesion and TCR molecules, contact with APC is prolonged such that sustained TCR:CD3 signals can propagate activation. A recent paper has suggested that 4 h is the minimum time required for resting, previously stimulated T cells to acquire an activation signal, while 10 h of stimulation is necessary for complete T-cell activation, including proliferation and cytokine production (114). The affinity of the peptide ligand has been shown to drastically affect the capacity of T cells to proliferate and secrete effector cytokine molecules including IFN γ , as APLs encompassing high, moderate, and low affinities have been shown to differentially alter T-cell activation following interaction with antigen-pulsed DCs (115). Specifically, low affinity ligands induce upregulation of T-cell activation markers in the absence of commitment to a full activation program characterized by proliferation and cytokine secretion. These results led to speculation that low affinity ligands fail in their capacity to decelerate T cells, leading to partial activation followed by anergy induction. In other studies, it has been shown that stimulation of the TCR with a low potency ligand increases NFATc expression, leading to an increase of IL-4 synthesis (116), while sequence analysis suggested an elongation in the TCR CDR3 loops expressed by antigen-specific Th2 clones compared with Th1 clones (117). Thus, T cells may be instructed to acquire a Th1 or Th2 phenotype from a very early stage of T-cell activation depending on TCR binding strength, which can be readily envisioned to impact CD3 signaling from both structural and biochemical studies.

Implications for TCR:cytokine receptor crosstalk

A recent series of reports suggest that the immunological synapse may play a role in the differentiation of CD4⁺ T-cell subsets, through the recruitment and activation of cytokine receptors and their immediate signaling intermediates (118,119). Specifically, ligation of TCR on naive CD4⁺ cells led to the recruitment of the IFN γ receptor (IFNGR1/2) and signal transducer and activator of transcription-1 (STAT1) to the immunological synapse. This was antagonized by IL-4 treatment via a STAT6-dependent mechanism. Cooperative signaling between the TCR:CD3 complex and cytokine receptors/signaling intermediates is an interesting proposal which fits nicely with previously reported results. Strikingly, Th2 cells exhibit unstable synapses, while in Th1 cells synapses are hyperstable (52). These observations are consistent with the morphological differences reported between Th1 and Th2 cells (57) and possibly due to the competing actions of PKC θ , a requisite signaling molecule for Th2 responses (120), and WASp (52). It is possible that TCR stimulation by default is associated with a recruitment of the IFNGR and STAT1 to the immunological synapse, wherein competition for signaling intermediates precludes recruitment and activation of the IL-4 receptor. However, under conditions of lower TCR affinity and altered CD3 signal strength, there is diminished or altered formation of the prototypical 'bull's eye' characteristic of Th1-type synapses, and there is instead formation of a multifocal synapse

characteristic of Th2 responses. In such a scenario, components of the JAK/STAT pathway are concomitantly activated by TCR ligation and may be accessible following activation of the IL-4R, as has been recently shown for the IFNGR (119). Although not yet examined, it remains possible that similar effects may be observed using other recently described skewing conditions for the generation of Th17 (121), Th9 (122), or even regulatory cells (123). Although there have been reports that Th1 and Th2 cells exhibit preferential usage of various intracellular signaling pathways, it is unknown whether phosphorylation of different ITAMs of the various CD3 subunits may alter the commitment of naive T cells to one lineage or another. Recent work from our laboratory (1) did suggest that phosphorylation of a single CD3 δ ITAM was sufficient to mediate cytokine secretion in the absence of a proliferative response. However, whether or not the capacity to activate particular ITAM sequences may alter the capacity of naive ITAM-mutant CD4⁺ T cells to differentiate in response to exogenous cytokines along defined lineages is unknown. The mechanism underlying the recruitment of IFNGR and STAT signaling proteins to the immunological synapse is unclear (119), so a role for various CD3 subunits and their preferential recruitment of adapter molecules cannot be excluded. Such investigations are certainly warranted.

Signaling for secretion

It is expected that T cells which are committed to activation and differentiation exhibit polarization of the microtubule organizing center (MTOC) and the secretory apparatus of the cell, towards the point of contact with APCs. This process has been attributed to dynein, through an interaction with adhesion and degranulation-promoting adapter protein (ADAP) (124) in a manner which requires an elevated level of localized diacylglycerol (DAG) (125). It is unknown if CD3 ITAM mutant mice are defective in the coupling of the TCR:CD3 signaling complex to the cytoskeleton; however, deficiencies in LAT:SLP-76 signaling and dysregulation of PLC γ or Vav recruitment are readily envisioned. Although cytokines are readily detected in cell culture supernatants derived from activated T cells expressing mutant CD3 ITAMs (1), it is currently unknown if there are any deficiencies in polarized secretion. Failure to relocalize the secretory apparatus towards the point of contact with APC may have detrimental effects on T-cell activation, particularly in light of recent findings which suggest cytokine receptor inclusion (118,119) and directed secretion of particular cytokines (126) within the immunological synapse. Recent evidence suggests that effector cytokines may be differentially sorted and secreted, such that cytokines including IL-2 and IFN γ are released from within the cSMAC of Th1 cells (126) in a manner dependent upon microtubule polarization, in agreement with a mechanism suggested previously (127). In contrast, TNF α exhibited multidirectional secretion (126). Similar results were obtained using Th2 effectors, wherein IL-10 was localized to the immunological synapse while IL-4 was observed to be secreted in a manner similar to TNF α . Interestingly, the chemokines macrophage inflammatory protein 1 α (MIP1 α) and RANTES (regulated upon activation, normal T-cell expressed, and presumably secreted) were also secreted in a multidirectional manner from CD4⁺ cells. Together, these results suggest that the targeted release of cytokines such as IL-2 or IFN γ may support an elevated local level of these key effector molecules, leading to enhanced T cell or APC activation, or possibly altered activation in the case of IL-10. In contrast, the multidirectional secretion of chemoattractant molecules or TNF α may serve a broader purpose to recruit other lymphocytes to the site of T-cell priming. Collectively, these and other recent studies (118,119) suggest that the formation of an immunological synapse induced by TCR:CD3 activation not only provides a support structure for classical T-cell signaling molecules but also facilitates crosstalk between these events and signaling pathways important for T-cell lineage differentiation. The dynamic inclusion or exclusion of cytokine receptors under conditions of hyper- or hypostable synapses has not been evaluated. However, it could be predicted that a hyperstable synapse

is amenable to the recruitment of the IFNGR:STAT1 signaling machinery, while the engagement of STAT6 may promote synapse disassembly leading to a Th2 phenotype. The relative contributions of the various CD3 ITAM subunits to immunological synapse formation and cytokine receptor recruitment are presently unknown, as are the contributions of TCR affinity in the context of scalable CD3 signaling, leading to T-cell activation and differentiation. An additional intriguing question is whether the relative strength of signal imparted by ITAM phosphorylation may have differential effects on the functional capacity of CD4⁺ versus CD8⁺ T cells, with respect to their requirements to secrete cytokines or release lytic granules (reviewed in 128). While CD8⁺ T cells quickly reorient the secretory apparatus and lytic granules towards the point of contact with a cell targeted for removal, activated CD4⁺ T cells target cytokine-containing vesicles to the immunological synapse with significantly delayed kinetics. Thus, it remains possible that perturbations in TCR signal strength may differentially influence early versus late events in T-cell function and activation depending upon cell lineage.

Role for ITAMs in the regulation of TCR:CD3 signaling

TCR signaling is negatively controlled by a plethora of regulatory mechanisms which are transcriptionally or post-translationally regulated, and which exhibit their effects on both developmental stages and during peripheral activation of mature T cells (reviewed in 129). A longstanding observation has been that T-cell activation results in downregulation of surface TCR expression, followed by degradation within the lysosomal compartment (50), in a manner which is likely due to several overlapping, non-exclusive mechanisms.

ZAP-70 can be phosphorylated by Lck on three tyrosine residues, which can impart either positive or negative regulation (reviewed in 76). The results of several studies suggested that Tyr292 phosphorylation was responsible for recruitment of the E3 ligase c-Cbl, thereby promoting internalization and degradation of the TCR complex via a ubiquitin-dependent mechanism (78,79,130). These observations were consistent with results obtained from ZAP-70 Y292F knockin mice which exhibit enhanced phosphorylation of the ZAP-70 substrates LAT and SLP-76 upon TCR activation (81). However, this mechanism does not appear to operate in developing thymocytes, while more recent results have cast doubt on the exact mechanism underlying the observed phenomenon (131). Recent results from our laboratory do however suggest a role for tonic ubiquitination of the CD3 complex in regulating TCR surface expression on developing thymocytes and subsequent selection fate (H Wang and D Vignali, unpublished observations).

The E3 ligases c-Cbl and Cbl-b have been determined to be key regulators of T-cell signaling (132–134). Several studies have shown that Cbl-b is a negative regulator of T-cell activation due to its ability to bind Vav. Deletion of Cbl-b is sufficient to uncouple the necessity for CD28 engagement in the production of IL-2 in response to TCR stimulation (132,135), which in turn may involve PI3K (136). Examination of other early parameters of T-cell activation revealed no differences or only slight enhancement of pZAP-70 (134), while analysis of downstream effector functions such as IL-2 or IFN γ production yielded mixed results. Nonetheless, Cbl-b represents a key negative regulator of T-cell activation, whose absence results in broad spectrum autoimmunity. Studies performed using c-Cbl and Cbl-b double knockout T cells indicated a failure to downregulate TCR expression following ligation (134), while further results support the observation that TCR downregulation is due to lysosomal degradation (45;50).

The contribution of a ubiquitination process in the development of autoreactive thymocytes or in the activation phenotype of T cells derived from ITAM mutant mice is presently unclear. It is conceivable that a TCR:CD3 complex with fewer phosphorylatable ITAMs

may insufficiently recruit adapter molecules such as Vav or Grb2 due to decreased ZAP-70 and LAT activation, thereby decreasing the amount of ubiquitinated proteins within the TCR signalosome while invoking the minimal signaling required to initiate activation and cytokine expression. Based on previous reports (132,133), it is possible that a ubiquitin deficiency, and hence defective TCR degradation, could lead to hyperproliferation under conditions of decreased ITAM signal strength. CD3 ITAM mutant T cells clearly exhibit defective proliferation (1), suggesting that (i) ubiquitination and degradation of the TCR:CD3 complex is sufficiently normal under these circumstances and the defect in proliferation lies elsewhere, or (ii) a defect in ubiquitination is secondary and of little consequence to a larger block in TCR recruitment of signaling intermediates occurring upstream of E3 ligase activity. Of note, lysine residues are of varied frequency among ITAM sequences of the CD3 subunits, with CD3 γ , CD3 ϵ , and the second and third ITAMs of CD3 ζ each containing a single lysine residue, while the CD3 δ and membrane proximal CD3 ζ ITAMs are devoid of an internal lysine located between the key tyrosine residues. Interestingly, our previous data suggested differing roles for the CD3 δ and CD3 γ chains, wherein ITAM mutant mice containing nine functional ITAMs and either a mutated δ or γ ITAM sequence, incapable of phosphorylation, resulted in an increased number of thymocytes in the former and not the latter. Whether or not this observed phenotype is related to the ubiquitination status of the CD3 complex and the δ subunit in particular or is due to differential recruitment of signaling molecules by the phosphorylated δ or γ ITAMs is a matter for investigation. However, the importance of c-Cbl in fine tuning the signaling capacity of developing thymocytes has been previously determined (137).

ITAM phosphorylation may govern recruitment of negative regulators

Deregulated signaling in CD3 ITAM mutant mice may be due in part to aberrant recruitment of negative regulators of TCR signaling. The importance of negative regulation is highlighted by Motheaten mice, wherein developing thymocytes and peripheral T cells are observed to be hyperproliferative in response to TCR stimulation. This defect has been attributed to a splice mutation yielding a frameshift mutation in the SHP-1 coding sequence (138). Conversely, overexpression of SHP-1 acts as a negative regulator of TCR activation (reviewed in 139). Interestingly, stimulation of the TCR with either agonist or antagonist, influences the negative or positive association of SHP-1 with the TCR:CD3 complex, respectively (65,140). Since a doubly phosphorylated ITAM contains tandem SH2 domains for the recruitment of the Syk family of PTKs, strong TCR stimulation leads to activation of ZAP-70 or Syk, thereby propagating an activation signal. In contrast, weak stimulation with a partial agonist results in incomplete phosphorylation of the CD3 ζ chain (64) and formation of a single SH2 domain capable of recruiting SHP-1. Although we have generated ITAM mutant mice wherein both tyrosines of the ITAM sequences have been selectively mutated (1), we cannot exclude the possibility that an ITAM rendered 'dead' by a Y to F mutation may have detrimental effects on the phosphorylation status of the remaining ITAMs, in a manner analogous to that shown for the CD3 ζ subunit which exhibits stepwise, partial progression of ITAM phosphorylation in response to stimulation (67). Thus, creation of a single rather than double SH2 domains, may preferentially recruit SHP-1 rather than ZAP-70 leading to an inactivation of Lck and abrogated T-cell activation. In summary, our understanding of how individual ITAMs may positively or negatively regulate various aspects of T-cell development and activation is in its infancy. However, building on the work of others, we speculate that the regulation of TCR:CD3 complex expression and signaling may be a complex process which includes mechanisms directly dependent upon ITAM sequence differences, capacity to undergo receptor-mediated phosphorylation, and the interaction of individual ITAMs with recruited signaling intermediates and adapter molecules. These will subsequently display various levels of regulation including

competitive binding with negative regulators such as SHP-1 or their targeted degradation via a ubiquitin-dependent process.

Concluding remarks and future questions

Our understanding of how T cells recognize antigen, become activated, and subsequently differentiate along one of an increasingly recognized number of T-cell lineages has seen immense progress in the 25 years since the identity of the TCR was confirmed. However, many fundamental questions remain, including why does the TCR:CD3 complex exhibit such complexity? This question had been difficult to address, and a fully comprehensive investigation of individual ITAM subunit function was largely impossible until the advent of our recently described retroviral approach for the generation of ‘retrogenic’ mice (1,141). This strategy has facilitated analyses of T-cell development and activation, wherein thymocytes or peripheral T cells exhibit altered capacities to initiate TCR signaling due to variability in the number and type of functional ITAMs (1). While this approach clearly indicated a role for ITAM number and location within the TCR:CD3 complex in both T-cell development and activation, differences in signal initiation, amplification, and diversification remain largely undefined. Considering the currently published data, we propose the following key areas which remain unresolved and represent exciting areas of future research: (i) Are all CD3 subunit cytoplasmic domains subject to similar regulatory mechanisms as recently reported for the CD3 ϵ chain, and does the location of individual CD3 molecules within the TCR complex contribute to the temporal regulation of ITAM phosphorylation? (ii) Do individual ITAMs exhibit differential affinities for signaling and adapter molecules, *in vivo*, and what (if any) are the differing contributions of ITAM subunits to initiation of a fully activated T-cell response? (iii) Does TCR stimulation of CD4⁺ and CD8⁺ T-cell lineages yield differences in signaling which can be dissected to the level of ITAM initiation, or are effector functions a combination of both TCR and cytokine receptor signaling events? (iv) Can differences in signaling requirements for T-cell development, peripheral T-cell activation, cytokine production and other T-cell function be discriminated on the basis of individual ITAM function? These are but a few questions worthy of investigation, and we speculate that deficiencies in T-cell activation under circumstances of decreased CD3 ITAM signal capacity may involve multiple, non-exclusive mechanisms. These may be readily envisioned due to the capacity of individual ITAM subunits to alter both early recruitment of signaling molecules and intermediates, or to influence the regulation of the TCR:CD3 complex by negative regulators. Additionally, ITAM signal strength may modulate distal TCR signaling events and possibly affect the downstream differentiation of such T cells via alterations in signaling molecules or cytokine receptors involved in lineage commitment.

Acknowledgments

DAAV is supported by the National Institutes of Health (NIH) (AI52199), a Cancer Center Support CORE grant (CA21765) and the American Lebanese Syrian Associated Charities (ALSAC).

Reference List

1. Holst J, et al. Scalable signaling mediated by T cell antigen receptor-CD3 ITAMs ensures effective negative selection and prevents autoimmunity. *Nat Immunol.* 2008; 9:658–666. [PubMed: 18469818]
2. Krogsaard M, Davis MM. How T cells ‘see’ antigen. *Nat Immunol.* 2005; 6:239–245. [PubMed: 15716973]
3. Call ME, Wucherpfennig KW. Common themes in the assembly and architecture of activating immune receptors. *Nat Rev Immunol.* 2007; 7:841–850. [PubMed: 17960150]

4. Weiss A. T cell antigen receptor signal transduction: a tale of tails and cytoplasmic protein-tyrosine kinases. *Cell*. 1993; 73:209–212. [PubMed: 8477442]
5. Hedrick SM, Cohen DI, Nielsen EA, Davis MM. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature*. 1984; 308:149–153. [PubMed: 6199676]
6. Malissen M, et al. Mouse T cell antigen receptor: structure and organization of constant and joining gene segments encoding the beta polypeptide. *Cell*. 1984; 37:1101–1110. [PubMed: 6611207]
7. Yanagi Y, Yoshikai Y, Leggett K, Clark SP, Aleksander I, Mak TW. A human T cell-specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains. *Nature*. 1984; 308:145–149. [PubMed: 6336315]
8. Dal Porto JM, Gauld SB, Merrell KT, Mills D, Pugh-Bernard AE, Cambier J. B cell antigen receptor signaling 101. *Mol Immunol*. 2004; 41:599–613. [PubMed: 15219998]
9. Kadlecsek TA, et al. Differential requirements for ZAP-70 in TCR signaling and T cell development. *J Immunol*. 1998; 161:4688–4694. [PubMed: 9794398]
10. Letourneur F, Klausner RD. Activation of T cells by a tyrosine kinase activation domain in the cytoplasmic tail of CD3 epsilon. *Science*. 1992; 255:79–82. [PubMed: 1532456]
11. Combadiere B, Freedman M, Chen L, Shores EW, Love P, Lenardo MJ. Qualitative and quantitative contributions of the T cell receptor zeta chain to mature T cell apoptosis. *J Exp Med*. 1996; 183:2109–2217. [PubMed: 8642321]
12. Love PE, Shores EW. ITAM multiplicity and thymocyte selection: how low can you go? *Immunity*. 2000; 12:591–597. [PubMed: 10894159]
13. Pitcher LA, van Oers NS. T-cell receptor signal transmission: who gives an ITAM? *Trends Immunol*. 2003; 24:554–560. [PubMed: 14552840]
14. Smith-Garvin JE, Koretzky G, Jordan MS. T cell activation. *Annu Rev Immunol*. 2009; 27:591–619.
15. Xu C, et al. Regulation of T cell receptor activation by dynamic membrane binding of the CD3 epsilon cytoplasmic tyrosine-based motif. *Cell*. 2008; 135:702–713. [PubMed: 19013279]
16. Germain RN. T-cell signaling: the importance of receptor clustering. *Curr Biol*. 1997; 7:R640–R644. [PubMed: 9368747]
17. Rudolph MG, Stanfield RL, Wilson IA. How TCRs bind MHCs, peptides, and coreceptors. *Annu Rev Immunol*. 2006; 24:419–466. [PubMed: 16551255]
18. Beddoe T, et al. Antigen ligation triggers a conformational change within the constant domain of the alpha beta T cell receptor. *Immunity*. 2009; 30:777–788. [PubMed: 19464197]
19. Irvine DJ, Purbhoo MA, Krogsgaard M, Davis MM. Direct observation of ligand recognition by T cells. *Nature*. 2002; 419:845–849. [PubMed: 12397360]
20. Valitutti S, Muller S, Cella M, Padovan E, Lanzavecchia A. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature*. 1995; 375:148–151. [PubMed: 7753171]
21. Krogsgaard M, Li QJ, Sumen C, Huppa JB, Huse M, Davis MM. Agonist/endogenous peptide-MHC heterodimers drive T cell activation and sensitivity. *Nature*. 2005; 434:238–243. [PubMed: 15724150]
22. Weiss A, Stobo JD. Requirement for the coexpression of T3 and the T cell antigen receptor on a malignant human T cell line. *J Exp Med*. 1984; 160:1284–1299. [PubMed: 6208306]
23. Kuhns MS, Davis MM, Garcia KC. Deconstructing the form and function of the TCR/CD3 complex. *Immunity*. 2006; 24:133–139. [PubMed: 16473826]
24. Gil D, Schamel WW, Montoya M, Sanchez-Madrid F, Alarcon B. Recruitment of Nck by CD3 epsilon reveals a ligand-induced conformational change essential for T cell receptor signaling and synapse formation. *Cell*. 2002; 109:901–912. [PubMed: 12110186]
25. Mingueau M, Sansoni A, Gregoire C, Roncagalli R, Aguado E, Weiss A, et al. The proline-rich sequence of CD3 epsilon controls T cell antigen receptor expression on and signaling potency in preselection CD4+CD8+ thymocytes. *Nat Immunol*. 2008; 9:522–532. [PubMed: 18408722]
26. Szymczak AL, et al. The CD3 epsilon proline-rich sequence, and its interaction with Nck, is not required for T cell development and function. *J Immunol*. 2005; 175:270–275. [PubMed: 15972658]

27. Tailor P, et al. The proline-rich sequence of CD3epsilon as an amplifier of low-avidity TCR signaling. *J Immunol.* 2008; 181:243–255. [PubMed: 18566390]
28. Takeuchi K, et al. Structural and functional evidence that Nck interaction with CD3epsilon regulates T-cell receptor activity. *J Mol Biol.* 2008; 380:704–716. [PubMed: 18555270]
29. Sigalov AB, Aivazian DA, Uversky VN, Stern LJ. Lipid-binding activity of intrinsically unstructured cytoplasmic domains of multichain immune recognition receptor signaling subunits. *Biochemistry.* 2006; 45:15731–15739. [PubMed: 17176095]
30. Delgado P, Fernandez E, Dave V, Kappes D, Alarcon B. CD3delta couples T-cell receptor signalling to ERK activation and thymocyte positive selection. *Nature.* 2000; 406:426–430. [PubMed: 10935641]
31. Werlen G, Hausmann B, Palmer E. A motif in the alphabeta T-cell receptor controls positive selection by modulating ERK activity. *Nature.* 2000; 406:422–426. [PubMed: 10935640]
32. Krogsgaard M, et al. Evidence that structural rearrangements and/or flexibility during TCR binding can contribute to T cell activation. *Mol Cell.* 2003; 12:1367–1378. [PubMed: 14690592]
33. Ma Z, Janmey PA, Finkel TH. The receptor deformation model of TCR triggering. *FASEB J.* 2008; 22:1002–1008. [PubMed: 17984179]
34. Aivazian D, Stern LJ. Phosphorylation of T cell receptor zeta is regulated by a lipid dependent folding transition. *Nat Struct Biol.* 2000; 7:1023–1026. [PubMed: 11062556]
35. Davis SJ, van der Merwe PA. The kinetic-segregation model: TCR triggering and beyond. *Nat Immunol.* 2006; 7:803–809. [PubMed: 16855606]
36. Dustin ML, Olszowy MW, Holdorf AD, Li J, Bromley S, Desai N, et al. A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts. *Cell.* 1998; 94:667–677. [PubMed: 9741631]
37. Dustin ML. The cellular context of T cell signaling. *Immunity.* 2009; 30:482–492. [PubMed: 19371714]
38. Sun ZY, Kim ST, Kim IC, Fahmy A, Reinherz EL, Wagner G. Solution structure of the CD3epsilon delta ectodomain and comparison with CD3epsilon gamma as a basis for modeling T cell receptor topology and signaling. *Proc Natl Acad Sci USA.* 2004; 101:16867–16872. [PubMed: 15557001]
39. Monks CR, Freiberg BA, Kupfer H, Sciaky N, Kupfer A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature.* 1998; 395:82–86. [PubMed: 9738502]
40. Springer TA. Adhesion receptors of the immune system. *Nature.* 1990; 346:425–434. [PubMed: 1974032]
41. Kupfer A, Singer SJ. The specific interaction of helper T cells and antigen-presenting B cells. IV. Membrane and cytoskeletal reorganizations in the bound T cell as a function of antigen dose. *J Exp Med.* 1989; 170:1697–1713. [PubMed: 2530300]
42. Dustin ML, Colman DR. Neural and immunological synaptic relations. *Science.* 2002; 298:785–789. [PubMed: 12399580]
43. Grakoui A, et al. The immunological synapse: a molecular machine controlling T cell activation. *Science.* 1999; 285:221–227. [PubMed: 10398592]
44. Ilani T, Vasiliver-Shamis G, Vardhana S, Bretscher A, Dustin ML. T cell antigen receptor signaling and immunological synapse stability require myosin IIA. *Nat Immunol.* 2009; 10:531–539. [PubMed: 19349987]
45. Lee KH, et al. The immunological synapse balances T cell receptor signaling and degradation. *Science.* 2003; 302:1218–1222. [PubMed: 14512504]
46. Lee KH, Holdorf AD, Dustin ML, Chan AC, Allen PM, Shaw AS. T cell receptor signaling precedes immunological synapse formation. *Science.* 2002; 295:1539–1542. [PubMed: 11859198]
47. Varma R, Campi G, Yokosuka T, Saito T, Dustin ML. T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster. *Immunity.* 2006; 25:117–127. [PubMed: 16860761]
48. Campi G, Varma R, Dustin ML. Actin and agonist MHC-peptide complex-dependent T cell receptor microclusters as scaffolds for signaling. *J Exp Med.* 2005; 202:1031–1036. [PubMed: 16216891]

49. Yokosuka T, et al. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. *Nat Immunol.* 2005; 6:1253–1262. [PubMed: 16273097]
50. Valitutti S, Muller S, Salio M, Lanzavecchia A. Degradation of T cell receptor (TCR)-CD3-zeta complexes after antigenic stimulation. *J Exp Med.* 1997; 185:1859–1864. [PubMed: 9151711]
51. Huang J, et al. CD28 plays a critical role in the segregation of PKC theta within the immunologic synapse. *Proc Natl Acad Sci USA.* 2002; 99:9369–9373. [PubMed: 12077322]
52. Sims TN, et al. Opposing effects of PKCtheta and WASp on symmetry breaking and relocation of the immunological synapse. *Cell.* 2007; 129:773–785. [PubMed: 17512410]
53. Dustin ML. T-cell activation through immunological synapses and kinapses. *Immunol Rev.* 2008; 221:77–89. [PubMed: 18275476]
54. Friedl P, den Boer AT, Gunzer M. Tuning immune responses: diversity and adaptation of the immunological synapse. *Nat Rev Immunol.* 2005; 5:532–545. [PubMed: 15999094]
55. Richie LJ, Ebert PJ, Wu LC, Krummel MF, Owen JJ, Davis MM. Imaging synapse formation during thymocyte selection: inability of CD3zeta to form a stable central accumulation during negative selection. *Immunity.* 2002; 16:595–606. [PubMed: 11970882]
56. Ebert PJ, Ehrlich LJ, Davis MM. Low ligand requirement for deletion and lack of synapses in positive selection enforce the gauntlet of thymic T cell maturation. *Immunity.* 2008; 29:734–745. [PubMed: 18993085]
57. Thauland TJ, Koguchi Y, Wetzel SA, Dustin ML, Parker DC. Th1 and Th2 cells form morphologically distinct immunological synapses. *J Immunol.* 2008; 181:393–399. [PubMed: 18566405]
58. Brossard C, et al. Multifocal structure of the T cell -dendritic cellsynapse. *Eur J Immunol.* 2005; 35:1741–1753. [PubMed: 15909310]
59. Samelson LE, Patel MD, Weissman AM, Harford JB, Klausner RD. Antigen activation of murine T cells induces tyrosine phosphorylation of a polypeptide associated with the T cell antigen receptor. *Cell.* 1986; 46:1083–1090. [PubMed: 2428504]
60. Irving BA, Weiss A. The cytoplasmic domain of the T cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways. *Cell.* 1991; 64:891–901. [PubMed: 1705867]
61. Chan AC, Iwashima M, Turck CW, Weiss A. ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. *Cell.* 1992; 71:649–662. [PubMed: 1423621]
62. Hatada MH, et al. Molecular basis for interaction of the protein tyrosine kinase ZAP-70 with the T-cell receptor. *Nature.* 1995; 377:32–38. [PubMed: 7659156]
63. Bu JY, Shaw AS, Chan AC. Analysis of the interaction of ZAP-70 and syk protein-tyrosine kinases with the T-cell antigen receptor by plasmon resonance. *Proc Natl Acad Sci USA.* 1995; 92:5106–5110. [PubMed: 7761456]
64. Madrenas J, Wange RL, Wang JL, Isakov N, Samelson LE, Germain RN. Zeta phosphorylation without ZAP-70 activation induced by TCR antagonists or partial agonists. *Science.* 1995; 267:515–518. [PubMed: 7824949]
65. Kersh EN, Kersh GJ, Allen PM. Partially phosphorylated T cell receptor zeta molecules can inhibit T cell activation. *J Exp Med.* 1999; 190:1627–1636. [PubMed: 10587353]
66. Dittel BN, Stefanova I, Germain RN, Janeway CA Jr. Cross-antagonism of a T cell clone expressing two distinct T cell receptors. *Immunity.* 1999; 11:289–298. [PubMed: 10514007]
67. van Oers NS, Tohlen B, Malissen B, Moomaw CR, Afendis S, Slaughter CA. The 21-and 23-kD forms of TCR zeta are generated by specific ITAM phosphorylations. *Nat Immunol.* 2000; 1:322–328. [PubMed: 11017104]
68. Isakov N, Wange RL, Burgess WH, Watts JD, Aebersold R, Samelson LE. ZAP-70 binding specificity to T cell receptor tyrosine-based activation motifs: the tandem SH2 domains of ZAP-70 bind distinct tyrosine-based activation motifs with varying affinity. *J Exp Med.* 1995; 181:375–380. [PubMed: 7528772]
69. Zenner G, Vorherr T, Mustelin T, Burn P. Differential and multiple binding of signal transducing molecules to the ITAMs of the TCR-zeta chain. *J Cell Biochem.* 1996; 63:94–103. [PubMed: 8891907]

70. Vely F, Nunes JA, Malissen B, Hedgecock CJ. Analysis of immunoreceptor tyrosine-based activation motif (ITAM) binding to ZAP-70 by surface plasmon resonance. *Eur J Immunol.* 1997; 27:3010–3014. [PubMed: 9394831]
71. Osman N, Turner H, Lucas S, Reif K, Cantrell DA. The protein interactions of the immunoglobulin receptor family tyrosine-based activation motifs present in the T cell receptor zeta subunits and the CD3 gamma, delta and epsilon chains. *Eur J Immunol.* 1996; 26:1063–1068. [PubMed: 8647168]
72. Graef IA, Holsinger LJ, Diver S, Schreiber SL, Crabtree GR. Proximity and orientation underlie signaling by the non-receptor tyrosine kinase ZAP70. *EMBO J.* 1997; 16:5618–5628. [PubMed: 9312021]
73. June CH, et al. Inhibition of tyrosine phosphorylation prevents T-cell receptor-mediated signal transduction. *Proc Natl Acad Sci USA.* 1990; 87:7722–7726. [PubMed: 2217205]
74. Barber EK, Dasgupta JD, Schlossman SF, Trevillyan JM, Rudd CE. The CD4 and CD8 antigens are coupled to a protein-tyrosine kinase (p56lck) that phosphorylates the CD3 complex. *Proc Natl Acad Sci USA.* 1989; 86:3277–3281. [PubMed: 2470098]
75. Veillette A, Bookman MA, Horak EM, Bolen JB. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. *Cell.* 1988; 55:301–308. [PubMed: 3262426]
76. Au-Yeung BB, et al. The structure, regulation, and function of ZAP-70. *Immunol Rev.* 2009; 228:41–57. [PubMed: 19290920]
77. Kong G, Dalton M, Bubeck WJ, Straus D, Kurosaki T, Chan AC. Distinct tyrosine phosphorylation sites in ZAP-70 mediate activation and negative regulation of antigen receptor function. *Mol Cell Biol.* 1996; 16:5026–5035. [PubMed: 8756661]
78. Lupher ML Jr, Songyang Z, Shoelson SE, Cantley LC, Band H. The Cbl phosphotyrosine-binding domain selects a D(N/D)XpY motif and binds to the Tyr292 negative regulatory phosphorylation site of ZAP-70. *J Biol Chem.* 1997; 272:33140–33144. [PubMed: 9407100]
79. Wang HY, et al. Cbl promotes ubiquitination of the T cell receptor zeta through an adaptor function of Zap-70. *J Biol Chem.* 2001; 276:26004–26011. [PubMed: 11353765]
80. Wu J, Zhao Q, Kurosaki T, Weiss A. The Vav binding site (Y315) in ZAP-70 is critical for antigen receptor-mediated signal transduction. *J Exp Med.* 1997; 185:1877–1882. [PubMed: 9151714]
81. Magnan A, et al. T cell development and T cell responses in mice with mutations affecting tyrosines 292 or 315 of the ZAP-70 protein tyrosine kinase. *J Exp Med.* 2001; 194:491–505. [PubMed: 11514605]
82. Sasahara Y, et al. Mechanism of recruitment of WASP to the immunological synapse and of its activation following TCR ligation. *Mol Cell.* 2002; 10:1269–1281. [PubMed: 12504004]
83. Gong Q, et al. Requirement for tyrosine residues 315 and 319 within zeta chain-associated protein 70 for T cell development. *J Exp Med.* 2001; 194:507–518. [PubMed: 11514606]
84. Weiss A, Imboden JB. Cell surface molecules and early events involved in human T lymphocyte activation. *Adv Immunol.* 1987; 41:1–38. [PubMed: 2961214]
85. Williams BL, et al. Phosphorylation of Tyr319 in ZAP-70 is required for T-cell antigen receptor-dependent phospholipase C-gamma1 and Ras activation. *EMBO J.* 1999; 18:1832–1844. [PubMed: 10202147]
86. Zhang W, Sloan-Lancaster J, Kitchen J, Tribble RP, Samelson LE. LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell.* 1998; 92:83–92. [PubMed: 9489702]
87. Zhang W, et al. Essential role of LAT in T cell development. *Immunity.* 1999; 10:323–332. [PubMed: 10204488]
88. Rudd CE. Adaptors and molecular scaffolds in immune cell signaling. *Cell.* 1999; 96:5–8. [PubMed: 9989491]
89. Bunnell SC, et al. T cell receptor ligation induces the formation of dynamically regulated signaling assemblies. *J Cell Biol.* 2002; 158:1263–1275. [PubMed: 12356870]
90. Finco TS, Kadlecsek T, Zhang W, Samelson LE, Weiss A. LAT is required for TCR-mediated activation of PLCgamma1 and the Ras pathway. *Immunity.* 1998; 9:617–626. [PubMed: 9846483]

91. Zhang W, Tribble RP, Samelson LE. LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation. *Immunity*. 1998; 9:239–246. [PubMed: 9729044]
92. Bubeck WJ, Fu C, Jackman JK, Flotow H, Wilkinson SE, Williams DH, et al. Phosphorylation of SLP-76 by the ZAP-70 protein-tyrosine kinase is required for T-cell receptor function. *J Biol Chem*. 1996; 271:19641–19644. [PubMed: 8702662]
93. Yablonski D, Kuhne MR, Kadlecik T, Weiss A. Uncoupling of nonreceptor tyrosine kinases from PLC-gamma1 in an SLP-76-deficient T cell. *Science*. 1998; 281:413–416. [PubMed: 9665884]
94. Bubeck WJ, et al. Regulation of PAK activation and the T cell cytoskeleton by the linker protein SLP-76. *Immunity*. 1998; 9:607–616. [PubMed: 9846482]
95. Hayashi K, Altman A. Protein kinase C theta (PKCtheta): a key player in T cell life and death. *Pharmacol Res*. 2007; 55:537–544. [PubMed: 17544292]
96. Acuto O, Michel F. CD28-mediated co-stimulation: a quantitative support for TCR signalling. *Nat Rev Immunol*. 2003; 3:939–951. [PubMed: 14647476]
97. Raab M, Pfister S, Rudd CE. CD28 signaling via VAV/SLP-76 adaptors: regulation of cytokine transcription independent of TCR ligation. *Immunity*. 2001; 15:921–933. [PubMed: 11754814]
98. Bi K, et al. Antigen-induced translocation of PKC-theta to membrane rafts is required for T cell activation. *Nat Immunol*. 2001; 2:556–563. [PubMed: 11376344]
99. Yokosuka T, et al. Spatiotemporal regulation of T cell costimulation by TCR-CD28 microclusters and protein kinase C theta translocation. *Immunity*. 2008; 29:589–601. [PubMed: 18848472]
100. Sun Z, et al. PKC-theta is required for TCR-induced NF-kappaB activation in mature but not immature T lymphocytes. *Nature*. 2000; 404:402–407. [PubMed: 10746729]
101. Love PE, et al. T cell development in mice that lack the zeta chain of the T cell antigen receptor complex. *Science*. 1993; 261:918–921. [PubMed: 7688481]
102. Lin SY, Ardouin L, Gillet A, Malissen M, Malissen B. The single positive T cells found in CD3-zeta/eta-/- mice overtly react with self-major histocompatibility complex molecules upon restoration of normal surface density of T cell receptor-CD3 complex. *J Exp Med*. 1997; 185:707–715. [PubMed: 9034149]
103. Dave VP, Cao Z, Browne C, Alarcon B, Fernandez-Miguel G, Lafaille J, et al. CD3 delta deficiency arrests development of the alpha beta but not the gamma delta T cell lineage. *EMBO J*. 1997; 16:1360–1370. [PubMed: 9135151]
104. Haks MC, Krimpenfort P, Borst J, Kruisbeek AM. The CD3gamma chain is essential for development of both the TCRalphabeta and TCRgammadelta lineages. *EMBO J*. 1998; 17:1871–1882. [PubMed: 9524111]
105. Malissen M, Gillet A, Ardouin L, Bouvier G, Trucy J, Ferrier P, et al. Altered T cell development in mice with a targeted mutation of the CD3-epsilon gene. *EMBO J*. 1995; 14:4641–4653. [PubMed: 7588594]
106. Negishi I, et al. Essential role for ZAP-70 in both positive and negative selection of thymocytes. *Nature*. 1995; 376:435–438. [PubMed: 7630421]
107. Cheng AM, Chan AC. Protein tyrosine kinases in thymocyte development. *Curr Opin Immunol*. 1997; 9:528–533. [PubMed: 9287188]
108. Palacios EH, Weiss A. Distinct roles for Syk and ZAP-70 during early thymocyte development. *J Exp Med*. 2007; 204:1703–1715. [PubMed: 17606633]
109. Love PE, Lee J, Shores EW. Critical relationship between TCR signaling potential and TCR affinity during thymocyte selection. *J Immunol*. 2000; 165:3080–3087. [PubMed: 10975819]
110. Sommers CL, et al. Function of CD3 epsilon-mediated signals in T cell development. *J Exp Med*. 2000; 192:913–919. [PubMed: 10993922]
111. Kersh EN, Shaw AS, Allen PM. Fidelity of T cell activation through multistep T cell receptor zeta phosphorylation. *Science*. 1998; 281:572–575. [PubMed: 9677202]
112. Pitcher LA, et al. The CD3 gamma epsilon/delta epsilon signaling module provides normal T cell functions in the absence of the TCR zeta immunoreceptor tyrosine-based activation motifs. *Eur J Immunol*. 2005; 35:3643–3654. [PubMed: 16259006]

113. van Stipdonk MJ, Lemmens EE, Schoenberger SP. Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nat Immunol.* 2001; 2:423–429. [PubMed: 11323696]
114. Huppa JB, Gleimer M, Sumen C, Davis MM. Continuous T cell receptor signaling required for synapse maintenance and full effector potential. *Nat Immunol.* 2003; 4:749–755. [PubMed: 12858171]
115. Skokos D, et al. Peptide-MHC potency governs dynamic interactions between T cells and dendritic cells in lymph nodes. *Nat Immunol.* 2007; 8:835–844. [PubMed: 17632517]
116. Brogdon JL, Leitenberg D, Bottomly K. The potency of TCR signaling differentially regulates NFATc/p activity and early IL-4 transcription in naive CD4+ T cells. *J Immunol.* 2002; 168:3825–3832. [PubMed: 11937535]
117. Boyton RJ, Zaccari N, Jones EY, Altmann DM. CD4 T cells selected by antigen under Th2 polarizing conditions favor an elongated TCR alpha chain complementarity-determining region 3. *J Immunol.* 2002; 168:1018–1027. [PubMed: 11801634]
118. Maldonado RA, Irvine DJ, Schreiber R, Glimcher LH. A role for the immunological synapse in lineage commitment of CD4 lymphocytes. *Nature.* 2004; 431:527–532. [PubMed: 15386021]
119. Maldonado RA, et al. Control of T helper cell differentiation through cytokine receptor inclusion in the immunological synapse. *J Exp Med.* 2009; 206:877–892. [PubMed: 19349465]
120. Marsland BJ, Soos TJ, Spath G, Littman DR, Kopf M. Protein kinase C theta is critical for the development of in vivo T helper (Th)2 cell but not Th1 cell responses. *J Exp Med.* 2004; 200:181–189. [PubMed: 15263025]
121. Zhou L, Littman DR. Transcriptional regulatory networks in Th17 cell differentiation. *Curr Opin Immunol.* 2009; 21:146–152. [PubMed: 19328669]
122. Dardalhon V, et al. IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(–) effector T cells. *Nat Immunol.* 2008; 9:1347–1355. [PubMed: 18997793]
123. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol.* 2008; 8:523–532. [PubMed: 18566595]
124. Combs J, Kim SJ, Tan S, Ligon LA, Holzbaur EL, Kuhn J, et al. Recruitment of dynein to the Jurkat immunological synapse. *Proc Natl Acad Sci USA.* 2006; 103:14883–14888. [PubMed: 16990435]
125. Quann EJ, Merino E, Furuta T, Huse M. Localized diacylglycerol drives the polarization of the microtubule-organizing center in T cells. *Nat Immunol.* 2009; 10:627–635. [PubMed: 19430478]
126. Huse M, Lillemeier BF, Kuhns MS, Chen DS, Davis MM. T cells use two directionally distinct pathways for cytokine secretion. *Nat Immunol.* 2006; 7:247–255. [PubMed: 16444260]
127. Morales-Tirado V, et al. Cutting edge: selective requirement for the Wiskott-Aldrich syndrome protein in cytokine, but not chemokine, secretion by CD4+ T cells. *J Immunol.* 2004; 173:726–730. [PubMed: 15240657]
128. Huse M, Quann EJ, Davis MM. Shouts, whispers and the kiss of death: directional secretion in T cells. *Nat Immunol.* 2008; 9:1105–1111. [PubMed: 18800163]
129. Acuto O, Di BV, Micheli F. Tailoring T-cell receptor signals by proximal negative feedback mechanisms. *Nat Rev Immunol.* 2008; 8:699–712. [PubMed: 18728635]
130. Balagopalan L, et al. c-Cbl-mediated regulation of LAT-nucleated signaling complexes. *Mol Cell Biol.* 2007; 27:8622–8636. [PubMed: 17938199]
131. Thien CB, Scaife RM, Papadimitriou JM, Murphy MA, Bowtell DD, Langdon WY. A mouse with a loss-of-function mutation in the c-Cbl TKB domain shows perturbed thymocyte signaling without enhancing the activity of the ZAP-70 tyrosine kinase. *J Exp Med.* 2003; 197:503–513. [PubMed: 12591907]
132. Bachmaier K, et al. Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. *Nature.* 2000; 403:211–216. [PubMed: 10646608]
133. Murphy MA, et al. Tissue hyperplasia and enhanced T-cell signalling via ZAP-70 in c-Cbl-deficient mice. *Mol Cell Biol.* 1998; 18:4872–4882. [PubMed: 9671496]

134. Naramura M, Jang IK, Kole H, Huang F, Haines D, Gu H. c-Cbl and Cbl-b regulate T cell responsiveness by promoting ligand-induced TCR down-modulation. *Nat Immunol.* 2002; 3:1192–1199. [PubMed: 12415267]
135. Chiang YJ, et al. Cbl-b regulates the CD28 dependence of T-cell activation. *Nature.* 2000 Jan 13;403:216–220. [PubMed: 10646609]
136. Fang D, Liu YC. Proteolysis-independent regulation of PI3K by Cbl-b-mediated ubiquitination in T cells. *Nat Immunol.* 2001; 2:870–875. [PubMed: 11526404]
137. Naramura M, Kole HK, Hu RJ, Gu H. Altered thymic positive selection and intracellular signals in Cbl-deficient mice. *Proc Natl Acad Sci USA.* 1998; 95:15547–15552. [PubMed: 9861006]
138. Lorenz U, Ravichandran KS, Burakoff SJ, Neel BG. Lack of SHPTP1 results in src-family kinase hyperactivation and thymocyte hyperresponsiveness. *Proc Natl Acad Sci UA.* 1996 Sep 3; 93(18):9624–9.
139. Lorenz U. SHP-1 and SHP-2 in T cells: two phosphatases functioning at many levels. *Immunol Rev.* 2009 Mar; 228(1):342–59. [PubMed: 19290938]
140. Stefanova I, Hemmer B, Vergelli M, Martin R, Biddison WE, Germain RN. TCR ligand discrimination is enforced by competing ERK positive and SHP-1 negative feedback pathways. *Nat Immunol.* 2003 Mar; 4(3):248–54. [PubMed: 12577055]
141. Holst J, Vignali KM, Burton AR, Vignali DA. Rapid analysis of T-cell selection in vivo using T cell-receptor retrogenic mice. *Nat Methods.* 2006 Mar; 3(3):191–7. [PubMed: 16489336]