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ITAM Multiplicity and Thymocyte Selection: How Low Can You Go?

Review

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ITAM Multiplicity in the Immune System

An intriguing feature of many receptors that participate in immune responses is the presence of multiple subunits and/or motifs that mediate signal transduction. One of the best studied of these signaling motifs is the ITAM (immune-receptor tyrosine-based activation motif) (Reth, 1989). Virtually all receptors that utilize ITAMs for signal transduction contain multiple copies of these motifs. These include some Fc receptors (Ravetch and Kinet, 1991), activating NK receptors (Lanier et al., 1998), PIR-A (paired immunoglobulin-like receptor-A) (Kubagawa et al., 1999), the B cell antigen receptor (BCR) (Kurosaki, 1999), and the T cell antigen receptor (TCR) (Klausner et al., 1990). Depending on the particular cell type and state of maturation, signals mediated through ITAMs can regulate cell survival, cell death, development, or effector functions.

ITAMs consist of semiconserved sequences of amino acids that contain two appropriately spaced tyrosines ($YXXL/I\ X_{6–8}\ YXXL/I$; where X denotes nonconserved residues) (Reth, 1989). Following receptor engagement, phosphorylation of ITAM tyrosine residues by Src family kinases represents one of the earliest events in the signaling cascade (reviewed in Weiss, 1993; Wange and Samelson, 1996; Rudd, 1999). In general, phosphorylation of both tyrosines within an ITAM (diphosphorylation) is thought to be essential for signaling, as this is required for efficient recruitment of the tandem SH2 domain containing protein tyrosine kinases Syk and ZAP-70 to the receptor complex. Activation of Syk and/or ZAP-70 results in the recruitment and phosphorylation of proteins that couple immune receptors to downstream signaling pathways.

While the importance of ITAMs for signal transduction is clear, it remains uncertain why immune receptors contain multiple ITAMs. In this review we discuss three possible mechanisms by which ITAM multiplicity may regulate signal transduction by immune receptors and examine how these regulatory mechanisms relate to TCR signaling and thymocyte selection.

Potential Regulatory Functions for ITAM Multiplicity

Perhaps the most straightforward function for ITAM multiplicity may be to facilitate signal amplification by increasing the local concentration of effector molecules (Figure 1A). ITAM-mediated signal amplification could result from simple quantitative effects of multiple ITAMs or could involve more complex mechanisms. For example, cooperative interactions between different ITAMs could promote the phosphorylation of additional ITAMs by the recruitment of tyrosine kinases, as has been described for signaling through FcεRI and the TCR (Lin et al., 1996; Ashe et al., 1999). Second, ITAM multiplicity may promote signal discrimination. For example, specific ITAMs may preferentially couple immune receptors to distinct downstream signaling pathways (Figure 1B). In this regard it is important to appreciate that although ITAM sequences are conserved, they are not identical, and hence may be able to associate with distinct molecules or associate with the same effectors with different affinities. Third, multiple ITAMs could potentially negatively regulate signaling by immune receptors, depending on the extent or specific pattern of ITAM phosphorylation (Figure 1C). For example, monophosphorylated ITAMs could directly recruit inhibitory proteins, such as phosphatases, to the receptor complex. The tyrosine phosphatases SHP-1, SHP-2, and SHIP associate with a sequence (YxxL) that closely resembles a monophosphorylated ITAM (referred to as an immune-receptor tyrosine-based inhibitory motif [ITIM]) present in several inhibitory coreceptors, including FcγRIIB, CD22, and KIRs. The recruitment of tyrosine phosphatases to coreceptors that contain ITIMs has been shown to negatively regulate signaling through ITAM-containing activating receptors (Vivier and Daeron, 1997). Alternatively, ITAMs could act to sequester effector molecules in their inactive forms, making such molecules unavailable to other receptors, as has been suggested for FcεRI (Torigoe et al., 1998). The mechanisms by which ITAM multiplicity may regulate immune function are not mutually exclusive, and each may be critical, depending on the cell type.

ITAM Multiplicity and the TCR

The TCR contains several distinct signaling subunits with up to ten ITAMs distributed among these chains. Although the clonotypic (TCRα and TCRβ) chains are required for antigen recognition, they do not directly participate in signal transduction. Instead, TCR signaling is mediated by the invariant chains of the TCR: CD3-γ, -δ, -ε, and ζ chain. Each of the CD3 components contains a single ITAM within its cytoplasmic tail, and because there are two CD3ε chains in each TCR complex, the CD3 chains collectively contribute four ITAMs to the TCR. In contrast, ζ chain, which exists in the TCR as a disulfide-linked dimer, contains three tandem ITAMs; therefore, the ζζ homodimer contributes six ITAMs to the TCR complex. Phosphorylation of the TCR-ITAMs by Lck and Fyn and the subsequent recruitment and activation of ZAP-70 results in phosphorylation of effector molecules such as the adaptor proteins LAT and SLP-76, which link the TCR to PLC-γ1, Grb-2/Sos, and PI3K, resulting in the activation of the calcium and MAP kinase pathways (van Leeuwen and Samelson, 1999).

The potential for signal regulation by multiple ITAMs is perhaps most critical in situations in which fine-tuning

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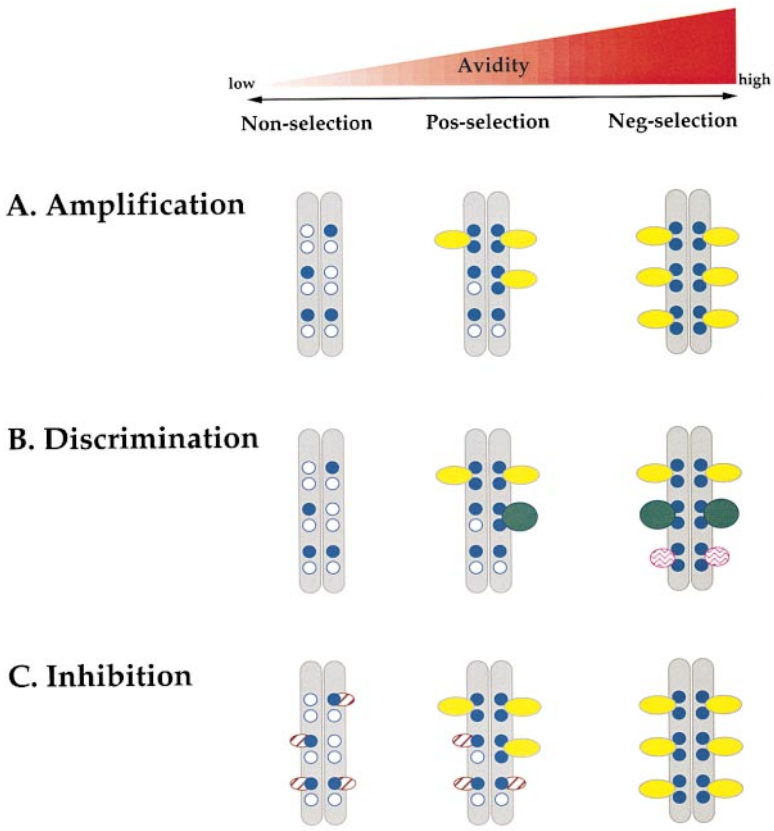

Figure 1. Possible Mechanisms by which Multiple ITAMs May Regulate Signaling by the TCR

Open circles, nonphosphorylated tyrosines; closed circles, phosphotyrosines. For simplicity, the TCR signaling apparatus is depicted as a ζ chain homodimer.

(A) Signal amplification. This model speculates that the extent of TCR-ITAM phosphorylation and the strength of TCR signal are directly related and are determined by the avidity of the TCR/MHC/ligand interaction.

(B) ITAM discrimination. In this model, individual ITAMs are speculated to recruit distinct activation complexes that can couple the TCR to different downstream signaling pathways. The extent of TCR-ITAM phosphorylation, determined by the avidity of the TCR-ligand interaction, determines the specific set of activation complexes recruited to the TCR. Although the figure depicts different effectors binding to individual ITAMs, discrimination could also operate through formation of distinct activation complexes, determined by the kinetics of the TCR/ligand interaction, without necessitating differential ITAM/effector interactions (see text).

(C) ITAM-mediated inhibition. This model speculates a negative regulatory role for ITAMs. For example, monophosphorylated ITAMs could sequester effector molecules in their inactive forms or directly recruit inhibitory proteins to the TCR (as shown). Inhibitory proteins that bind to monophosphorylated ITAMs could be displaced by activation complexes upon complete phosphorylation of the ITAM or could compete with proteins that bind preferentially to diphosphorylated ITAMs. Alternatively, the multiple ITAM tyrosine residues may serve as a sink or decoy that prevent or delay the formation of any single diphosphorylated ITAM.

of the signaling response is required. The complex process by which immature T cells undergo selection in the thymus clearly represents such a situation.

Thymocyte Selection

The TCR repertoire expressed by mature T cells is largely devoid of autoreactivity and is restricted to recognizing foreign peptides in the context of self-MHC molecules. In contrast, the TCR repertoire of immature thymocytes is vast, largely due to V(D)J recombination. The process of removing overt self-reactivity while imparting self-MHC restriction to the mature TCR repertoire is thought to operate via selective processes regulated primarily by the affinity/avidity of the TCR for thymic ligands (self-MHC and self-peptide) and the ensuing TCR-mediated signals (reviewed in Jameson et al., 1995; Sebzda et al., 1999). Because V(D)J recombination generates a random assortment of TCRs, the majority of immature $CD4^+CD8^+$ (DP) thymocytes will express TCRs lacking specificity for self-ligands (self-MHC + self-peptide) in the thymus, fail to receive signals required for survival, and die by a process termed "death by neglect." The remaining thymocytes undergo one of two antithetical fates, both of which paradoxically require TCR recognition of thymic ligands. Thymocytes expressing TCRs that have a relatively high avidity for thymic ligands receive signals that result in their deletion (physically or functionally deleted from the T cell repertoire). On the other hand, thymocytes expressing TCRs that have a lower avidity for thymic ligands receive quantitatively or qualitatively distinct signals that allow for their survival and eventual development to mature, functional, single-positive ($CD4^+CD8^-$ or $CD4^-CD8^+$) T cells.

ITAM Multiplicity and TCR Signal Amplification

The affinity of a TCR for its selecting ligand appears to be extremely low, and it is difficult to envision how such interactions might achieve an activation threshold sufficient to rescue them from death by neglect. An explanation that may reconcile this finding lies in the potential of multiple TCR ITAMs to amplify signals originating from low affinity/avidity interactions. One approach to assessing the role of ITAM multiplicity in thymocyte selection has involved the construction of mice selectively lacking individual TCR signal transducing chains or their ITAMs and examining the effect of these genetic alterations on thymocyte selection.

In depth analysis of mice lacking endogenous ζ chain has pointed to an important role for ITAM multiplicity and signal amplification during thymocyte selection. In the absence of ζ chain, TCR surface expression is markedly reduced (Liu et al., 1993; Love et al., 1993; Malissen et al., 1993; Ohno et al., 1993). In addition, $\zeta^{-/-}$ mice contain very few mature thymocytes and peripheral T cells, suggesting an impairment in positive selection. When the specificity of the TCRs expressed by these mature T cells was examined, the cells were found to be autoreactive, suggesting that negative selection was

also impaired (Lin et al., 1997). Because surface TCR expression is extremely low in ζ chain null mice, it was not possible to directly evaluate the role of ζ chain ITAMs on thymocyte selection in these mice. Therefore, $\zeta^{-/-}$ mice were reconstituted with transgenes encoding ζ chain variants containing 3, 2, 1, or 0 ITAMs (Shores et al., 1994, 1997a, 1997b). These experiments demonstrated that the efficiency of both positive and negative selection correlated directly with the number of ζ chain ITAMs present in the TCR complex. These studies are also consistent with the idea that multiple ζ chain ITAMs act in a quantitative manner to regulate TCR signaling during thymocyte selection.

Mice selectively lacking the CD3 components of the TCR (CD3-γ, -δ, -ε) have also been generated (Malissen et al., 1995; Dave et al., 1997; DeJarnette et al., 1998; Haks et al., 1998). In the absence of one or more CD3 proteins, TCR assembly is markedly impaired, resulting in extremely low or absent TCR surface expression. Therefore, it was not possible to directly assess the role of CD3 ITAMs on selection. A clear interpretation of the role of CD3 ITAMs in selection awaits the construction of mice that express mutant proteins that lack functional ITAMs, as in the experiments with ζ variant mice described above.

ITAM Multiplicity and Signal Discrimination

Interestingly, relatively small differences in affinity/avidity of TCR-ligand interactions can adjudicate the decision to live or die, suggesting that the signals resulting from slight differences in avidity must be precisely regulated during thymocyte selection (Alam et al., 1996). Indeed, the need for precise control of TCR signaling is highlighted when one considers that, ultimately, positive and negative selection require activation or induction of specific effector proteins such as members of the bcl-2/bcl-XL family for positive selection (Linette et al., 1994) and Nur 77/Nor-1 and caspases for negative selection (Calnan et al., 1995; Zhou et al., 1996; Alam et al., 1997; Cheng et al., 1997; Clayton et al., 1997; Hettman et al., 1999; Izquierdo et al., 1999).

If specific sets of effectors regulate the choice between positive and negative selection, it might be predicted that specific signaling pathways downstream of the TCR become activated. Indeed, several recent experiments support the notion that distinct signaling pathways mediate positive and negative selection. Data obtained from studies employing dominant-negative proteins, constitutively active kinases, and pharmacological agents have implicated the p21ras-raf-MKK1-ERK signaling pathway in positive selection but not in negative selection (Swan et al., 1995; Alberola-Ila et al., 1996a, 1996b; O'Shea et al., 1996; Swat et al., 1996; Sharp et al., 1997; Sugawara et al., 1998; Pages et al., 1999). Whether the association of ERK activation with positive selection reflects a qualitative or quantitative difference in the signaling response compared with negative selection remains controversial, as the levels of ERK activation required for positive selection may be lower than those required for negative selection and therefore more sensitive to blockade or augmentation (Shao et al., 1999). Similarly, a specific role for calcium-mediated signals in positive selection is also controversial, as its importance in negative selection appears to be a function of TCR-ligand binding strength and the consequent amplitude and oscillary patterns of the calcium flux (Vasquez et al., 1994; Wang et al., 1995; Kane and Hedrick, 1996; Mariathasan et al., 1998; Freedman et al., 1999). In contrast to positive selection, experiments employing specific pharmacological inhibitors and retroviral transfer systems indicate that the MKK6-p38 and JNK signaling pathways may be specifically involved in negative selection. (Rincon et al., 1998; Sugawara et al., 1998; Sabapathy et al., 1999). Together, these data support the notion that the outcome of thymocyte selection can be regulated by both qualitative and quantitative differences in TCR signaling responses.

The multiple ITAMs of the TCR may regulate signals during selection qualitatively by activating distinct downstream signaling pathways. Indeed, in vitro studies indicate that individual TCR ITAMs may preferentially bind to different molecules or the same molecules with distinct affinities (Isakov et al., 1995; Osman et al., 1995, 1996; Rozdzial et al., 1995). Hence, phosphorylation of specific ITAMs may lead to the preferential recruitment of different activation complexes to the TCR and the induction of different downstream signaling pathways. However, in vivo experiments in which mice expressing ζ chain variants possessing different subsets of TCR ITAMs have failed to find evidence of ITAM specificity (van Oers et al., 1998). Indeed, stimulation of thymocytes from mice that express ζ chains lacking all ITAMs were found to contain increased levels of phosphorylated CD3 chains compared with those expressing a wild-type (three ITAM) ζ chain. These data indicate that the CD3 ITAMs may partially compensate for the loss of ζ chain ITAMs and that ζ chain ITAMs probably do not play a specific role in thymocyte selection (van Oers et al., 1998). Moreover, the efficiency of thymocyte selection in H-Y TCR transgenic mice appeared similar in ζ chain variant mice regardless of whether their ζ chain contained only the membrane proximal or membrane distal ζ chain ITAM (Shores et al., 1997b). While these studies performed to date do not support a role for distinct ITAMs in selectively mediating signaling pathways required for positive versus negative selection, further experiments are needed to dissect the contribution of each TCR ITAM to selection.

On the other hand, it may not be necessary to invoke qualitative differences in the signaling responses regulating positive and negative selection if quantitative differences in molecules such as transcription factors can control thymocyte fate. For example, low levels of specific transcription factors may be sufficient to activate genes controlling positive selection, whereas higher quantities of the same factors may be required to activate genes promoting negative selection. In this respect, the potential of multiple ITAMs to amplify small initial differences in signal intensity could be critical, as the difference in the affinity of a TCR for its positively and negatively selecting ligand can be relatively small.

ITAM Multiplicity and Signal Inhibition

The multiple ITAM configuration of the TCR may also provide a means by which the TCR can transmit negative signals, thereby influencing the outcome of the selection process. Indeed, several recent experiments suggest that partially phosphorylated ITAMs may play a role in regulating signaling by the TCR. This idea initially arose from the discovery that stimulation of the TCR with "altered ligands" induces only partial phosphorylation of ζ chain (p21) and the recruitment of nonphosphorylated (inactive) ZAP-70. In contrast, stimulation of the TCR

with agonist ligands results in more extensive phosphorylation of ζ chain (p23), which leads to the recruitment and subsequent activation of ZAP-70 (Sloan-Lancaster et al., 1994; Madrenas et al., 1995). Studies in which thymocytes were stimulated with positively and negatively selecting ligands revealed that stimulation with either ligand resulted in the formation p23 phospho-ζ and phosphorylated ZAP-70. However, the levels of both p23 phospho-ζ and ZAP-70 were higher when negatively selecting ligand was used to stimulate cells (Smyth et al., 1998). These data are consistent with the idea that quantitative differences in ζ chain phosphorylation may regulate the outcome of selection. However, to date studies have only been performed on bulk populations of thymocytes. Thus, it is possible that qualitative differences in ζ chain phosphorylation patterns may promote positive versus negative selection, but such a conclusion will require examination at the single cell level.

It has also been proposed that differential ζ chain phosphorylation may serve to negatively regulate TCR signaling. In this regard, experiments performed with transfected cell lines indicate that p21-ζ contains monophosphorylated ITAMs, whereas p23-ζ contains one or more diphosphorylated ITAMs (Kersh et al., 1998). The implications of these data are intriguing, as recent studies have suggested a distinct role for monophosphorylated ζ chain ITAMs in initiating inhibitory signals (Kersh et al., 1999). A possible mechanism by which monophosphorylated ITAMs may serve a negative regulatory function may be to recruit inhibitory molecules such as tyrosine phosphatases to the TCR complex (Figure 1C). As previously discussed, the tyrosine phosphatase SHP-1 has been shown to associate with a sequence resembling a monophosphorylated ITAM (ITIM) and to negatively regulate BCR signaling (Vivier and Daeron, 1997). SHP-1 is also expressed in thymocytes, and recent results obtained with mice either lacking SHP-1, as in the mutant strain *motheaten*, or in mice overexpressing a dominant-negative form of the protein suggest that SHP-1 can function to negatively regulate signaling by the TCR and influence thymocyte selection (Carter et al., 1999; Johnson et al., 1999; Plas et al., 1999; Zhang et al., 1999).

Another possible explanation for the negative regulatory function of monophosphorylated ITAMs may lie in their potential to sequester activating molecules that are present in limiting amounts, thereby rendering these molecules inaccessible to other TCRs. For example, low-avidity TCR-ligand interactions may result in the generation of predominantly monophosphorylated ITAMs that could then sequester ZAP-70 in its inactive form. Indeed, this phenomenon has been demonstrated in mast cells, where engagement of some IgE receptors by weak ligands inhibited signal transduction by other IgE receptors engaging higher affinity ligands (Torigoe et al., 1998). Finally, the multiple tyrosine residues within a single TCR complex may act as a "sink," decreasing the possibility that diphosphorylation of any one ITAM will occur. Conceivably, such a mechanism could function to safeguard against activation by inappropriate stimuli.

Kinetic Models of TCR Signaling and ITAM Multiplicity

The kinetic proofreading model and the subsequently proposed kinetic discrimination model (McKeithan, 1995; Rabinowitz et al., 1996) have interesting implications for thymocyte selection and the role of ITAM multiplicity in this process. These models propose that for those responses regulated by a cascade of biochemical signaling events, such as those initiated by engagement of TCR, small differences in initial signaling events can be magnified through the subsequent events of the cascade. Moreover, they suggest that the probability of transversing each of the individual events in the pathway and successfully arriving at the penultimate event depends on the dwell time of the receptor with its ligand. Because the affinity of the TCR for ligand is closely related to the off rate of the interaction, ligands with different affinities for receptor are likely to have different TCR dwell times. Interpreting these models in relation to thymocyte selection leads to the hypothesis that high-affinity TCR/ligand interactions result in a dwell time that allows complete progression of the signaling pathway and the formation of late activation complexes, resulting in negative selection. In contrast, low-affinity interactions with short dwell times, such as those that mediate positive selection, would result in premature attenuation of the signaling cascade with accumulation of early complexes but not late activation complexes. Early activation complexes need not be inert and could have the potential to regulate distinct downstream activation pathways. Indeed, recent data have correlated positive and negative selection with different TCR-ligand dwell times (Williams et al., 1999).

Kinetic models of TCR signaling also have important ramifications for the regulatory mechanisms proposed for multiple ITAMs. The extent of ITAM phosphorylation, and thus the degree of signal amplification, would predictably be regulated by the dwell time of the TCR-ligand interaction. Further, the kinetics of TCR-ligand interactions could also influence the selective activation of downstream signaling pathways. For example, the duration of the TCR-ligand interaction might specify the particular subset of TCR ITAMs that becomes phosphorylated, thereby influencing which signaling pathways become activated. Alternatively, a requirement for preferential ITAM/effector associations need not be invoked if early and late activation complexes (described above) are capable of coupling the TCR to distinct signaling pathways. The TCR could thus respond to different ligands (such as those mediating positive or negative selection) by activating defined subsets of downstream signaling pathways depending on the TCR-ligand dwell time, even if all ITAMs bind the same effector molecules with similar affinities. Finally, the duration of TCR-ligand interactions could influence the outcome of selection by regulating the ratio of diphosphorylated versus monophosphorylated ITAMs. The induction of primarily monophosphorylated ITAMs, resulting from the short dwell time of low-avidity interactions, could directly serve to negatively regulate activation signals and selection by a variety of mechanisms, including sequestration and phosphatase recruitment. Moreover, the multiple tyrosine residues, rather than ITAM multiplicity per se, may indirectly regulate TCR signaling by reducing the probability that two tyrosines within a single ITAM (required for activation) could be phosphorylated during the dwell time of a TCR-ligand interaction.

Evolutionary Implications

The structural complexity of the TCR suggests that strong selective advantages must underlie the formation and maintenance of its configuration. Although TCR

subunit/ITAM multiplicity may be important for regulating mature T cell responses, experiments to date have failed to reveal such a role. For example, while ζ-ITAMs appear critical for selection of the T cell repertoire, they are not essential for T cell activation and effector responses (Shores et al., 1997b; Ardouin et al., 1999). Instead, we propose that the evolutionary advantage conferred by a TCR repertoire selected on the basis of low-affinity/avidity interactions to self-ligands drove the evolution of a receptor that contains multiple signaling motifs.

In support of this idea are data from the analysis of T cells that reside in the epithelium of the intestine (i-IELs). A subset of i-IELs (consisting of both $\alpha\beta TCR^+$ and $\gamma\delta TCR^+$ cells) express TCRs that include dimers composed of ζ chain and a structurally and functionally related family member, FcεR1γ (Fcγ), which contains only a single ITAM (Guy-Grand et al., 1994). Significantly, these T cells develop independently of the thymus and do not undergo selection in the same manner as thymocytes, as they include cells that express autoreactive TCRs (Rocha et al., 1992). In contrast, thymically derived T cells do not express Fcγ (Shores et al., 1998). Moreover, although Fcγ can restore TCR expression and thymocyte and T cell development in ζ-deficient mice, both positive and negative selection are impaired (Shores et al., 1997a). The ζ and Fcγ genes are closely linked in both mouse and humans (Küster et al., 1990). We speculate that ζ may have arisen by gene duplication of Fcγ in response to the requirement for a TCR subunit that contains multiple ITAMs. The restricted expression of ζ (T cells, human NK cells) contrasted with the much broader expression pattern of Fcγ (T cells, NK cells, mast cells, macrophages) and the utilization of Fcγ by several different receptor types suggests that Fcγ represents the original prototype of the ζ family molecules. Clustering of the genes encoding CD3-γ, -δ, and -ε likewise points to a common ancestral origin of these TCR subunits (Gold et al., 1987; Letourneur et al., 1989).

The importance of ITAM multiplicity for TCR function is also underscored by the structural and functional conservation of the TCR signal transducing subunits throughout evolution. For example, although mammalian and avian lineages diverged over 300 million years ago, the chicken ζ chain also contains 3 ITAMs and can restore TCR function in mouse cells lacking endogenous ζ chain (Gobel and Bolliger, 1998).

The ability to generate a TCR repertoire that is skewed toward recognizing self-MHC yet is largely depleted of autoreactive cells requires a precisely controlled mechanism of ligand recognition and signal transduction. The evolution of the TCR complex, with its multiple signaling subunits and activation motifs that enable formation of distinct activation complexes and signal amplification, appears ideally suited to support the development of this immune recognition system. However, the full details of how multiple signaling subunits and ITAMs of the TCR mechanistically regulate and facilitate the process of thymocyte selection await further elucidation. Understanding “how low you can go” both in terms of ITAM number and ITAM/effector specificity will be critical for understanding thymocyte selection.

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