Published in final edited form as:

Trends Immunol. 2014 July ; 35(7): 311–318. doi:10.1016/j.it.2014.05.003.

Fine-tuning T cell receptor signaling to control T cell development

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Abstract

T cell development from immature CD4⁺CD8⁺ double-positive (DP) thymocytes to the mature CD4 or CD8 single-positive (SP) stage requires proper T cell receptor (TCR) signaling. The current working model of thymocyte development is that the strength of the TCR-mediated signal – from little-or-none, through intermediate, to strong – received by the immature cells determines whether they will undergo death by neglect, positive selection, or negative selection, respectively. In recent years, several developmentally regulated, stage-specifically expressed proteins and miRNAs have been found that act like fine-tuners for signal transduction and propagation downstream of the TCR. This allows them to govern thymocyte positive selection. Here, we summarize recent findings on these molecules and suggest new concepts of TCR positive-selection signaling.

Keywords

T cell development; signaling; thymocyte; Themis; Tesp1; voltage-gated sodium channel; miR-181

Emerging fine-tuning molecules in T cell development During the process of negative selection in central immunological tolerance, immature T cells (i.e., thymocytes) that respond strongly to self MHC peptide (MHCp) through their TCRs are effectively eliminated from the pool of conventional mature T cells. Negative selection avoids generalized T cell aggression towards healthy tissue, while preserving a naïve T cell pool available to generate T cell responses against foreign MHCp when summoned to fight infection or malignant cell transformation [1]. Fulfilling the latter task is only ensured via the survival during thymocyte development, of immature T cells that recognize self MHCp

with low-to-moderate strength, in a process termed positive selection. Positive selection generally precedes negative selection in the thymus, signaling survival of the selected thymocytes and enabling these cells to distinguish foreign antigen in the context of their own self-MHC (i.e., MHC restriction). Remarkably, these two contrasting developmental outcomes, as well as the activation and differentiation process of mature T cells in response to foreign antigens, are all managed by a TCR signaling machinery of qualitatively similar composition [2]. The essential difference is in the interpretation of signals of different strength leading to antagonistic gene expression and metabolic changes that predispose to survival and differentiation, or that trigger cell death. How these diverse roles are achieved by the TCR in different cellular contexts, and indeed how these contexts affect TCR signaling, remains unclear.

The TCR signaling machine is dynamically assembled from at least three membrane-resident modules that are physically segregated at steady-state but which coalesce upon binding of TCR to MHCp with adequate affinity [3–5]. The TCR recognition module (TCR $\alpha\beta$ -CD3 $\gamma\epsilon\delta\epsilon\zeta$ 2) governs MHCp recognition and the association of the protein tyrosine kinase (PTK) zeta-chain-associated protein kinase of 70 kDa (ZAP70) with the intracellular immunoreceptor-tyrosine-based activation motifs (ITAMs) of CD3 molecules. These are phosphorylated by the Src family PTK lymphocyte-specific protein tyrosine kinase (Lck). The Src kinase module is in charge of regulating the activity of the PTKs Lck and Fyn, and ensures the TCR activation threshold. Finally, the diversification/regulatory module rapidly forms highly cooperative multiprotein complexes that fine tune the amplitude and duration of signal propagation along several pathways. The ZAP70, Lck, and Fyn PTKs and the membrane-associated linker of activated T cells/SH2 domain containing leukocyte protein of 76 kDa (LAT/SLP67) scaffold complex together govern this critical signaling step. These signals trigger the activation of multiple biochemical pathways and the increase in cytosolic free Ca²⁺ concentration (Figure 1).

Part of the problem in understanding TCR-dependent signaling in thymocyte development is that many of the biochemical and imaging studies of TCR signaling have been performed with mature T cells or tumor lines, whereas thymocyte development has mostly been studied at the genetic level. Moreover, those signaling experiments performed on thymocytes have mainly used antibody cross-linking, rather than a natural ligand, to stimulate the cells. Thus, our understanding of signaling processes in thymocyte development suffers from the entanglement of interpretations based on these different methodologies. In the past 30 years the main players in the signaling cascades have been identified, but only relatively recently have we started to identify molecules with more subtle roles that can distinguish signal strengths to give different responses for positive versus negative selection. Several genes involved in controlling proximal TCR signaling in thymocyte selection processes are found to be differentially expressed during thymocyte development, and Ca²⁺ signaling during thymocyte development is differentially ‘wired’ compared to that in mature T cells [6]. The idea is emerging that developmentally regulated, stage-specific expression of molecules that finely tune the TCR signal transduction and propagation processes govern positive selection. Here, we review these recent findings and suggest new concepts in TCR positive selection signaling.

Specialized signaling regulation for thymocyte positive selection

Thymocytes are more sensitive than mature T cells to low-affinity ligands [7,8]. This has biological consequences, because it allows weakly binding self-MHCps to readily trigger thymocyte positive selection without incurring the undesirable activation of mature T cells in the periphery. This relatively weak stimulation of thymocytes does not lead to overt activation that would induce negative selection or lineage deviation into one of the agonist-selected T cell subsets. Agonist-selected cells include the TCR $\alpha\beta^+$ CD8 $\alpha\alpha$ -expressing intraepithelial lymphocytes, Natural killer (NK)T cells, and thymus-derived T regulatory cells; all of which require interaction with higher-affinity ligands than are required for positive selection of conventional naïve CD4 $^+$ or CD8 $^+$ T cells (agonist selection is comprehensively reviewed in [9]). Because of this intertwined complexity and subtlety, it is reasonable to expect that specialized signaling regulators exist in thymocyte-positive selection to help translate signals into different cell fates [2].

Recent studies have identified proteins and miRNAs that seem to fit into this paradigm of specialized regulators of TCR signaling operating during thymocyte development. For example, the adaptors thymocyte-expressed molecule involved in selection (Themis) [10–14] and thymocyte-expressed, positive selection-associated-1 (Tespa1) [15], a voltage-gated Na $^+$ channel (VGSC) [16] (Figure 1), and miR-181 [17] are all important in signaling during positive selection. They are expressed at relatively high levels and/or in a restricted fashion in late double negative (DN) and immature TCR $^{\text{lo}}$ DP thymocytes, and are reduced or lacking in mature SP thymocytes or T cells. Their ablation results in defective transition of thymocytes from DP to SP and consequently to impaired mature T cell development, particularly of the CD4 $^+$ lineage. Additionally, although the adaptor growth factor receptor-bound protein 2 (GRB2) and the phosphatase SHP1 [Src homology region 2 (SH2) domain-containing phosphatase-1; PTPN (protein tyrosine phosphatase, non-receptor type)6] do not fall purely into the category of fine-tuning molecules for signaling, their interactions with other proteins point to this function. It is unclear at the moment whether the mode of action of all these actors is independent of each other, or whether they are inter-related. Below, we review recent evidence on the functions of these molecules in thymocyte development, placing these findings in the context of our current understanding of the biochemical mechanisms underlying positive and negative selection.

Themis negatively regulates the initial steps of the TCR signaling cascade

Themis is expressed exclusively in the T cell lineage, with expression levels being highest in DP thymocytes [10–13]. It is a member of a small gene family conserved throughout vertebrate evolution (Box 1). In biochemical studies using thymocytes and T cell lines, it was found that Themis is tyrosine-phosphorylated immediately after TCR crosslinking [10,18,19], by Lck and possibly ZAP70 [20]. Themis binds constitutively to GRB2 [11–13,18], its poly-proline region interacting with the C-terminal SH3 domain of GRB2 [20]. Themis also co-immunoprecipitates with other molecules of the LAT/SLP76 signaling module.

We initially described that Themis-deficient immature thymocytes had slightly reduced Ca $^{2+}$ flux and levels of phosphorylation of the mitogen-activated kinase (MAP) kinase

extracellular signal-regulated kinase (ERK), compared to wild type, after antibody crosslinking of TCR and co-receptors [10]. Decreased ERK activation upon anti-TCR-CD3 antibody stimulation was also observed in Jurkat T cells where Themis expression was reduced by siRNA targeting [18]. Other investigators were unable to discern any change in TCR signaling in thymocytes from Themis-deficient mice [11–13]. Thus, this first wave of analysis of Themis function by our group and others was remarkably uninformative and controversial. Neither the subtle TCR signaling defect [10], nor the various minor changes in cell survival and metabolism [11,12] noted by others seemed sufficient to explain the severely impaired T cell development phenotype in Themis-deficient mice.

Although historically widely used as a convenient surrogate, crosslinking antibody has a much higher affinity than MHCp and typically engages the CD3 ϵ chains instead of the TCR $\alpha\beta$ dimer. Pre-selection thymocytes have a low activation threshold and undergo positive selection by responding to MHCp of relatively low affinity (i.e., weak agonists and antagonists [21–23]), whereas antibody stimulation leads to apoptosis [24,25]. DP thymocytes respond to a continuum of MHCp affinities and are able to sense the affinity threshold at which positive selection (cell survival and differentiation), tips over by a digital switch to an opposite cell fate (cell death) [23]. Activating antibodies might, therefore, overload the signaling response and thus be unsuitable to reveal subtle signaling defects in thymocytes. We therefore re-examined TCR-proximal signaling in wild type and Themis-deficient OT-I TCR transgenic DP pre-selection thymocytes using specific MHCp known to trigger positive or negative selection. Agonist MHCp (H-2K^b presenting SIINFEKL peptide), variants of the agonist peptide with defined TCR binding strength and selection outcome, as well as endogenous positively selecting peptides are well characterized for the OT-I model [21,23]. High-affinity MHCp tetramers – the strong agonists that cause negative selection – showed little or no difference in TCR signaling (e.g., TCR-proximal phosphorylation events, elevation of intracellular calcium, and ERK activation) between Themis-deficient and sufficient DP thymocytes [26]. By contrast, low-affinity MHCp tetramers with positive selecting peptides induced stronger LAT and phospholipase C (PLC) γ 1 phosphorylation, Ca²⁺ influx, nuclear factor of activated T cells (NFAT) nuclear translocation, ERK activation, and intracellular localization of phosphorylated ERK (p-ERK) in Themis-deficient as compared to wild type pre-selection thymocytes [26]. Themis-deficient thymocytes responded to positive-selecting ligands in a similar manner to Themis-sufficient cells responding to higher-affinity ligands [26]. These results indicated that Themis is a negative regulator of TCR-proximal signaling and suggest that there is a shift to a lower-affinity threshold for agonist or negative selection in the absence of Themis.

Pre-selection *Themis*-deficient OT-I thymocytes show increased apoptosis in response to relatively low-affinity ligands compared to wild type [26]. Thymocyte development in the absence of Themis was significantly restored in mice deficient for both Themis and proapoptotic Bcl-2-like protein 11 (Bim), a proapoptotic protein required for negative selection [27]. The implication is that Themis acts in a somewhat similar manner to the ‘death-dampening’ effect of Schnurri-2 (Shn2) [28], a member of a family of zinc-finger proteins involved in morphogenesis in invertebrates, and in bone, adipocyte, and T cell development in vertebrates [29]. Like Themis, Shn2 was initially reported to have a role in

positive selection, because *Shn2*-deficient mice have a much reduced number of post-selection DP or SP thymocytes [30]. Recent data demonstrate that, in fact, it acts by inhibiting activation-induced cell death in response to positive selecting signals [28]. Unlike Themis, however, expression of *Shn2* is not restricted to the T cell lineage and it acts much further downstream in the TCR-dependent signaling cascade, apparently at the level of activation of the mitochondrial cell death pathway, although the mechanism is unclear [28]. Thus for both Themis and *Shn2*, the effect of the loss-of-function is increased negative selection in response to normal positive-selecting stimuli.

SHP1 regulation of thymocyte selection signaling: a role for Themis

SHP1 is a versatile cytoplasmic protein tyrosine phosphatase (PTPase) that has been implicated as a negative regulator in many signaling pathways including receptor tyrosine kinases, cytokine receptors, and antigen receptors [31,32]. In TCR signaling it acts on Lck [17,33], and reportedly on ZAP70 and SLP76 [31,34]. SHP1 acts in a negative feedback loop upon TCR signaling in experiments using antigen presenting cells (APC) pulsed with agonist or antagonist peptides to stimulate mature T cells [33]. These experiments showed that SHP1 worked by inhibiting Lck activity in response to antagonist, but not agonist ligands. SHP1 was unable to inhibit Lck after agonist stimulation because of positive feedback from ERK that phosphorylated Ser59 of Lck, blocking SHP1 from interacting with Lck [33]. By analogy, in developing thymocytes, SHP1 would be expected to turn off responses to positive-selecting ligands, but not to negative-selecting ligands. Multiple studies show that SHP1 sets the TCR-signaling threshold between positive and negative selection, using both SHP1-deficient ‘moth-eaten’ mice and dominant-negative forms of SHP1 [35–38]. However, a recent study using Cre recombinase gene under transcriptional control of elements of the CD4 gene (CD4-Cre) to induce conditional deletion of the *Ptpn6* gene, removing SHP1 in thymocytes from the earliest stage of DP, showed no obvious differences in positive or negative selection relative to wild type cells [39]. However, this study did not assess any possible redundancy in PTPase function, for example, through activity of Src homology region 2 domain-containing phosphatase-2 (SHP2). Although SHP2 appears to have positive roles in some pathways [31,32], it has a negative regulatory role in TCR signaling when associated with microclusters of the co-inhibitory receptor programmed death-1 (PD-1) [40]. Defective thymocyte development in SHP1 germline-deficient mice is the result of both impaired TCR signaling and severe inflammation, so this postulated functional redundancy between SHP1 and SHP2 and/or other PTPases may not be sufficient to rescue thymocyte development in these mice. At steady-state, SHP1 activity is inhibited by an intramolecular interaction of the N-terminal Src homology region 2 (N-SH2) domain obstructing the catalytic site [31]. There is also evidence that tyrosine phosphorylation of the SHP1 C-terminal tail plays a role in its function by binding to the adaptor protein GRB2 [41], and that this interaction is required for SHP1-mediated regulation of cytokine receptor signaling. However, these data were obtained using transfected cell lines, and the relevance of this interaction to thymocyte development remains undetermined. SHP2 is by no means the only PTPase that could potentially substitute for SHP1 in the CD4-Cre SHP1^{fl/fl} mice. PTPN22 and dual-specificity phosphatase (DUSP)22 can also dephosphorylate active Lck in TCR signaling in mature T

cells [42,43]. Their expression in mice carrying a conditional allele of SHP1 has not been investigated.

Interestingly, we found that basal SHP1 phosphorylation as well as TCR stimulation-induced SHP1 activation were much reduced or abolished in Themis-deficient thymocytes compared to wild type cells, suggesting potential Themis and SHP1 interaction [26]. Immunoprecipitations from pre-selection OT-I thymocytes confirmed binding between Themis and SHP1 (Figure 1). In light of the dispensable role of SHP1 in thymocyte development [39], we examined Themis and SHP2 interaction, finding that SHP2 can also bind to Themis. Although it remains to be determined whether SHP2 is a normal partner of Themis in thymocyte development, this observation argues for potential redundancy between SHP1 and SHP2 in thymocyte development. Thus, these studies point to the negative signaling role of Themis being mediated by SHP1 recruitment to the proximity of the TCR signalosome. This signaling alteration appears not to affect a particular TCR signaling pathway but globally reduces TCR signal-propagation, likely from Lck/ITAM onward.

Multiple roles of GRB2 in T cell development

GRB2 is a relatively small protein with one central SH2 domain flanked by two SH3 domains (Figure 1, insert). A study investigating GRB2 haploid-insufficient mice showed its importance for thymocyte negative, but not positive, selection [44]. A recent study used the Lck-Cre transgene to delete GRB2 specifically in pre-selection thymocytes, finding severe defects in both negative and positive selection, with few mature SP cells developing [45]. This result is perhaps not surprising considering the multiple interactions of GRB2 in TCR signaling, including binding to the Ras-guanine nucleotide exchange factor Son of Sevenless (SOS), protein tyrosine phosphatase SHP1, the adaptor molecule Tspal1 (see below), and Themis (where GRB2-binding was required for Themis recruitment to LAT and its phosphorylation [20]). Biochemically, anti-TCR and CD4 crosslinking of GRB2-deficient thymocytes showed reduced CD3 ζ , LAT, and PLC γ 1 phosphorylation, Ca²⁺ signaling, and MAP kinases C-Jun N-terminal kinase (JNK) and p38 activation. However, rather counter-intuitively given the importance of GRB2–SOS and RasGRP (which depends on PLC γ 1) in activating the Ras/ERK MAP kinase pathway, there was no alteration in Ras/ERK signaling. The authors concluded that GRB2 is a positive regulator of TCR-proximal signaling in thymocytes [45]. These data need to be reconciled with those from Themis- and Tspal1-deficient mice (see below), but it is possible that the controversy was simply because too-strong stimulants (antibody crosslinking) were used in the mechanistic studies and thus overshadowed the possibility of GRB2 acting as a negative regulator. Alternatively, a Ras-independent ERK signaling pathway has been reported in mature T cells, and this may also function in developing thymocytes. For this Ras-independent ERK signaling pathway and the roles of SOS and RasGRP in T cell development, readers are encouraged to read the recent elegant review by Kortum and colleagues [46]. GRB2 has a complicated and somewhat promiscuous binding pattern. Although a relatively small protein with limited functional domains, GRB2 interacts with multiple positive and negative regulatory proteins. However it is highly unlikely that GRB2 could bind all its potential partners at the same time. As GRB2 dimers have been shown to act functionally in signaling [47], such dimers

may allow bridging of several GRB2 partners at the same time. It is possible that preferential binding of some molecules to GRB2, with or without GRB2 dimerization, could occur only under positive or negative selection conditions.

Ca²⁺ signaling in positive selection: roles for VGSC and Tespa1

Despite their importance for store-operated Ca²⁺ entry (SOCE) in mature T cells, the components of the Ca²⁺ release activated Ca²⁺ (CRAC) channel, stromal interaction molecule 1 (STIM1) and calcium release-activated calcium modulator (ORAI), are not required for positive selection in thymocyte development [6,48], although the CRAC channel is required for development of agonist-selected cells [6]. However, it is well established that positive selection requires a sustained if rather gentle Ca²⁺ signal, whereas negative selection induces a stronger signal [23]. Thus, it was unclear how calcium signaling in positive selection was controlled if not by the CRAC channel. The discovery of a thymocyte-expressed VGSC formed from the proteins encoded by *Scn4b* and *Scn5a* [16] may help to answer this question. Lo and co-workers found that *Scn4b* expression was increased in immature thymocytes stimulated with a positive selecting ligand, but downregulated in response to a negative-selecting stimulus [16]. SCN4B is the regulatory subunit of the VGSC, with SCN5A being the pore-forming subunit. VGSC activity in excitable cells (neurons and muscle cells) is regulated by phosphorylation of SCN5A by several kinases relevant to T cell biology, including Fyn and protein kinase C. In thymocytes, inhibition of VGSC by tetrodotoxin (which blocks Na⁺ entry into the SCN5A channel) or *Scn5a* siRNA knockdown, or by a soluble form of the extracellular part of SCN4B, inhibited both the sustained Ca²⁺ influx and positive selection, with a stronger effect on CD4 SP generation than that of CD8 [16]. When the VGSC is ectopically expressed in mature T cells, it endows them with the sensitivity to respond to a low-affinity, positive-selecting ligand to which they normally do not show any response. The reason for stronger effect on CD4 SP generation than on CD8 is unclear, but the simplest explanation may be that selection into the CD4 compartment requires more sustained signaling than does CD8 generation [49], and that the sustained calcium influx resulting from VGSC is therefore more crucial to CD4 SP generation [16]. Thus, the VGSC appears to provide a mechanism for the sustained but weak calcium signal during positive selection, and to be important in the ability of immature thymocytes to respond to weak ligands [16]. The mechanistic link between sodium channel activity and calcium entry in thymocytes, however, remains enigmatic. For more information regarding ion channels in lymphocyte development and function, including the possibility for crosstalk between different ion channels, please see [50].

Tespa1 is another recently described protein that also fits into the paradigm of molecules specialized to regulate signaling in thymocyte positive selection [15]. It binds GRB2, likely through its predicted SH3-domain binding sites, and shows regulated expression in thymocyte development, being most strongly expressed in DP cells. Tespa1 appears to act in the TCR signaling cascade in the formation of the LAT signalosome, and its absence results in deficient calcium and ERK signaling pathways (as measured after antibody crosslinking) [15]. Tespa1 is homologous to K_i-Ras-induced actin-interacting protein (KRAP, also called sperm-specific antigen 2, Ssa2) [51]. KRAP is expressed in epithelial cells and interacts

with the inositol-1,4,5-trisphosphate (IP₃) receptor on the endoplasmic reticulum (ER), where it maintains the localization of the IP₃ receptor at the ER and thus regulates Ca²⁺ release from the ER store [52,53]. A region of KRAP including two adjacent phenylalanine residues that are required for this function [54] is conserved in Tespa1, suggesting that Tespa1 might regulate IP₃ receptor activity and therefore ER Ca²⁺ release in thymocytes [51]. It is now known that Tespa1 indeed interacts with the IP₃ receptor in T cells, and that the adjacent phenylalanine residues shared with KRAP are required for the interaction [55]. Tespa1 action may therefore be regulated by the TCR signaling cascade, in association with components of the LAT signalosome. It is unclear if it is also associated with the IP₃ receptor on the ER at this point in the signaling cascade, or whether it is translocated to the ER after stimulation. However, it is notable that KRAP is associated with IP₃ receptor at regions of the ER close to cell–cell contact areas [52]. This could suggest a similar localization of Tespa1 at the immunological synapse, and therefore an association with the LAT signalosome during TCR stimulation. The data suggest that Tespa1 is important in regulating ER Ca²⁺ store release, and thus regulation of SOCE. Interestingly, Tespa1 phosphorylation was recently shown to be initiated in response to SOCE rather than to ER store release itself [56]. However, given the data on VGSC, and the importance of the CRAC channel (and therefore SOCE?) only with agonist-selecting ligands, it may be premature to fit these data into the context of positive selection.

Tuning of TCR sensitivity by miRNAs

TCR sensitivity to MHCp ligands can also be tuned during thymocyte development by altering the amount of miRNAs, short noncoding RNAs that regulate gene expression at the post-transcriptional level by targeting specific mRNAs for degradation or translational repression. One such miRNA is miR-181a. Its expression correlates with T cell sensitivity, being high at the DP stage, downregulated after TCR signaling, and lowest in differentiated mature T cells [17,57]. Inhibition of miR-181a impairs both positive and negative selection in *in vitro* thymic development [57]. This is due to an altered TCR-signaling threshold, because inhibition of miR-181a results in maturation of autoreactive CD4 cells specific for moderate-affinity, negatively selecting, self peptides [17]. miR-181a increases TCR signal strength by repressing expression of several phosphatases that negatively regulate TCR signaling networks: DUSP5 and DUSP6 (dephosphorylate serines and tyrosines on ERK), and SHP2 and PTPN22 (negative regulators of TCR signaling) [17]. miR-181a reduces negative feedback on TCR signaling, causing higher basal tyrosine phosphorylation of ERK, and Ser59 phosphorylation of Lck. Although miR-181a does not directly target SHP1, by augmenting Lck phosphorylation at Ser59 it inhibits SHP1-targeting of Lck. However, the exact role of miR-181a in thymocyte development remains controversial, unsurprisingly for a molecule with the potential to regulate expression of thousands of proteins. Conditional deletion of miR-181ab1 at early stages of thymic development reduce total thymocyte numbers, but do not affect the percentages of conventional TCRαβ thymic compartments [58]. This decrease in total thymic cellularity is attributed to the role of miR-181a and miR-181b in regulation of phosphoinositide 3-kinase (PI3K) signaling and cellular metabolism, by targeting and downregulating phosphatase and tensin homolog (PTEN) [58]. No major TCR-signaling defects are observed in thymocytes deficient in miR-181a1 and miR-181b1 [58]. However, only antibody stimulation of TCR polyclonal thymocytes has

been reported, and this method may not reveal potential differences in TCR signaling in response to physiologically relevant MHCp ligand-binding affinity.

Positive selection and peripheral T cell responsiveness

The concept of TCR signal-tuning during thymocyte development can also be applied to a more nuanced view of positive selection, where the strength of TCR binding to positively selecting MHCp ligand dictates the subsequent functionality of the selected T cells. A recent study compared CD4 T cells from two mouse lines transgenic for structurally different TCRs binding the same antigen with comparable affinity, but presumably positively selected on different MHCp [59]. Unexpectedly, these T cells differed in interleukin (IL)-2 production and ERK phosphorylation in response to TCR ligation or stimulation with PMA plus ionomycin (which bypasses TCR-proximal signaling). The more-responsive T cells display higher levels of basal CD3 phosphorylation and cell-surface CD5, suggesting that they receive stronger signals from self-MHCp during thymic selection and/or in the periphery [59]. The changes in IL-2 responsiveness and CD5 levels are already observed at the CD4 SP stage, suggesting that strength of TCR binding to self-MHCp during positive selection alters the basal responsiveness of the selected T cells [59]. This attractive model of fine-tuning of T cell responses will have to be verified by identification of positively selecting peptides for the two TCRs, and demonstration that the peptide selecting the more responsive T cells binds the TCR with higher affinity. Identification of selecting ligands for the two TCRs will also help elucidate the molecular mechanism behind the selection-induced alterations in basal responsiveness. CD5 plays some role in reducing basal responsiveness, because both TCR transgenic lines show higher responses to phorbol myristate acetate (PMA)/ionomycin in the absence of CD5 [59]. However, CD5-mediated inhibition is not sufficient to reduce the basal responsiveness, because CD5 expression levels have been shown to correlate with higher responsiveness in T cells [59,60]. Given the role of negative regulators of signaling in thymic development, it would be interesting to determine whether changes in expression levels, activation status and/or localization of PTPases, especially those involved in regulation of ERK signaling, such as DUSP5 and DUSP6, contribute to the setting of basal T cell responsiveness level in the thymus.

TCR signal strength during thymocyte selection is modulated by expression of several different molecules. Themis and miR-181a act as, respectively, negative and positive regulators of TCR signal strength by modulating activity of cellular phosphatases. Themis and Tespa1 interact with GRB2 and form part of the LAT signalosome. Tespa1 and SCN4B regulate Ca^{2+} downstream of TCR signals. Themis, Tespa1, SCN4B, and miR-181a are expressed at high levels in pre-selection DP thymocytes, and their expression is reduced in peripheral cells, as expected for molecules with unique thymocyte-intrinsic roles in thymic selection.

Concluding remarks

Cell fate decision making in developing thymocytes depends critically on signaling input from TCR upon its interaction with selecting self-MHCp. The molecular mechanism underlying the increased sensitivity of pre-selection thymocytes to weak ligands remains

poorly understood. Recent research has identified multiple thymocyte-specific regulatory mechanisms, each partly responsible for the unique orchestration of TCR signaling in the thymus. Even after three decades of extensive study, TCR signaling and T cell development are still fascinating research areas. This is partially because of the very complicated nature of TCR signaling, but also because of recent unexpected discoveries. This highlights the importance for researchers not only to search for new players, but also, as studies on Themis using MHCp rather than antibody crosslinking have shown, to revisit the roles of some old players in more physiological experimental situations. Finally, we would argue that a child is not a small adult, and equally a thymocyte is not just simply an immature T cell. It deserves its own more physiologically relevant experimental system in addition to traditional classical approaches.

Acknowledgments

This work was supported by the National University of Singapore and National Institutes of Health grants GM065230 and DK094173 to N.R.J.G., and Wellcome Trust Grant WT094296MA and EU-FP7 'Sybilla' Grant 201106 to O.A.

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Box 1. The Themis gene family

Themis (Themis1) is the prototype of a three-member family in vertebrates [10–12]. Themis2 [induced by contact to basement membrane 1 (ICB1)] is similar in its sequence and in the relative lack of knowledge of its function. It is expressed in the B cell and macrophage/dendritic cell lineages, and has been shown to have a scaffolding function in lipopolysaccharide (LPS)-activated TLR signaling in a macrophage cell line, where it supports tumor necrosis factor (TNF) α production but not cyclo-oxygenase (COX)2 or IL-6 expression [61]. Themis2 is phosphorylated soon after stimulation and interacts with GRB2, similar to Themis1 [19]. It was recently tested for its ability to function in lieu of Themis1 when expressed as a transgene in Themis-deficient developing thymocytes. It is indeed able to support full development of thymocytes in place of Themis1 [19]. However, Themis2 is normally not expressed in T lineage cells. Thus, although Themis2 is not redundant with Themis1, it can function to perform at least some of the same duties. Themis3 (9130404H23Rik) is expressed in the gut but nothing is known of its function. A new predicted globular protein domain, CABIT (for cysteine-containing, all- β in Themis), was recently defined [11]. Proteins containing CABIT domains are found in several invertebrate phyla as far back in evolution as Cnidarians, and other vertebrate proteins with single CABIT domains have also been identified [11]. Clearly there is much scope for future research on the other members of the Themis family.

Figure 1.

Proteins affecting signaling during thymocyte positive selection. During thymocyte development, upon TCR interaction with self-MHCp complex, a cascade of signaling events is triggered. This process includes a series of tyrosine phosphorylations mediated by protein tyrosine kinases (e.g. Lck and ZAP-70), sequential signaling complex formation mediated by scaffold proteins (e.g., LAT and SLP-76), and more downstream diversified signaling pathways (e.g., ERK and NFAT). Over the past 30 years, many molecules important for thymocyte positive selection were discovered, characterized, and put in the context of the signaling network shown here. Recently, some newly identified molecules were found to be specifically involved in thymocyte positive selection (i.e., Themis, Tespa1, and VGSC). These each serve different functions in signaling in positive selection, acting with other molecules that may have a more general role in T cell signal transduction and negative selection. Insert. Model for GRB2-Themis-SHP1 complex association with LAT. Upon TCR stimulation during thymocyte positive selection, the scaffold protein LAT is tyrosine phosphorylated at multiple sites, which serve as the docking sites for many SH2-domain-containing molecules. Among these LAT-binding molecules, the adaptor protein GRB2 has a central SH2 domain flanked by two SH3 domains. Recent studies showed that Themis binds the C-terminal SH3 domain of GRB2 via its proline-rich region, whereas SHP1 binds the SH2 domain of GRB2 via its C-terminal phosphotyrosines. Thus GRB2 brings Themis and SHP1 together, and Themis is required for activity of SHP1. Abbreviations: DAG,

diacylglycerol; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; Gads, GRB2-related adapter protein 2; GRB2, Growth factor receptor-bound protein 2; IP3, inositol 1,4,5-trisphosphate; IP3R, IP3 receptor; Itk, interleukin-2-inducible T cell kinase; LAT, linker of activated T cells; Lck, lymphocyte-specific protein tyrosine kinase; MHCp, MHC peptide; NF- κ B, nuclear factor- κ B; NFAT, nuclear factor of activated T cells; PIP2, Phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PTP, protein tyrosine phosphatase; SH2, Src homology region 2 domain; SH3, Src homology region 3 domain; SHP1, Src homology region 2 domain-containing phosphatase-1; SLP-76, SH2 domain containing leukocyte protein of 76kDa; TCR, T cell receptor; Tespa1, thymocyte-expressed, positive selection-associated-1; Themis, thymocyte-expressed molecule involved in selection; VGSC, voltage-gated sodium channel; ZAP-70, zeta-chain-associated protein kinase of 70 kDa.