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CD8 T Cell Sensory Adaptation Dependent on TCR Avidity for Self-Antigens

Maria-Elena Marquez,² Wilfried Ellmeier,§ Vanesa Sanchez-Guajardo,† Antonio A. Freitas,† Oreste Acuto,* and Vincenzo Di Bartolo*

Adaptation of the T cell activation threshold may be one mechanism to control autoreactivity. To investigate its occurrence in vivo, we engineered a transgenic mouse model with increased TCR-dependent excitability by expressing a Zap70 gain-of-function mutant (ZAP-YEEI) in postselection CD8 thymocytes and T cells. Increased basal phosphorylation of the Zap70 substrate linker for activation of T cells was detected in ZAP-YEEI-bearing CD8 T cells. However, these cells were not activated, but had reduced levels of TCR and CD5. Moreover, they produced lower cytokine amounts and showed faster dephosphorylation of linker for activation of T cells and ERK upon activation. Normal TCR levels and cytokine production were restored by culturing cells in the absence of TCR/spMHC interaction, demonstrating dynamic tuning of peripheral T cell responses. The effect of avidity for self-ligand(s) on this sensory adaptation was studied by expressing ZAP-YEEI in P14 or HY TCR transgenic backgrounds. Unexpectedly, double-transgenic animals expressed ZAP-YEEI prematurely in double-positive thymocytes, but no overt alteration of selection processes was observed. Instead, modifications of TCR and CD5 expression due to ZAP-YEEI suggested that signal tuning occurred during thymic maturation. Importantly, although P14 × ZAP-YEEI peripheral CD8 T cells were reduced in number and showed lower Ag-induced cytokine production and limited lymphopenia-driven proliferation, the peripheral survival/expansion and Ag responsiveness of HY × ZAP-YEEI cells were enhanced. Our data provide support for central and peripheral sensory T cell adaptation induced as a function of TCR avidity for self-ligands and signaling level. This may contribute to buffer excessive autoreactivity while optimizing TCR repertoire usage.


The mature TCR repertoire is shaped by positive and negative selection processes resulting from interactions with self-peptide/MHC (spMHC) during thymic differentiation. A repertoire emerges that is sufficiently biased toward self-MHC recognition to ensure T cell survival, but is deprived of those TCRs whose high avidity for spMHC may lead to cellular activation and autoimmunity (1, 2). However, thymic negative selection may be leaky, as suggested by the detection in the peripheral T cell pool of self-reactive cells (2, 3), which are thought to be curbed by various mechanisms, including deletion, anergy, phenotypic skewing, or regulatory T cells (reviewed in Ref. 4). A conceptual framework to explain how self-tolerance is maintained was put forth in the tunable activation threshold hypothesis by Grossman and Paul (5). They proposed that although T cells are activated when TCR ligation induces intracellular signals of sufficient speed and amplitude, subthreshold stimuli (e.g., generating slow or low amplitude TCR signals) may lead to a gradual modification of responsiveness to subsequent stimuli. For example, autoreactive T cells that escaped central deletion or normally selected T cells facing changes in spMHC concentration when leaving the thymus may modify the TCR threshold to levels compatible with survival without activating the T cell (6, 7). This principle may apply to thymocyte maturation (8) during which TCR signaling can indeed be adjusted, e.g., by CD5 expression (9–11). At the molecular level, adaptation may operate by changing levels of expression and/or intrinsic activity of some excitation- and de-excitation-inducing factors, such as activating and inhibitory receptors and/or kinases and phosphatases (7).

A change in expression of CD5 that negatively regulates signaling through the TCR (9) is the best-known example of TCR tuning signaling, yet it cannot entirely explain central and peripheral modifications of T cell responsiveness (10–13). Moreover, although the effects of spMHC deprivation on T cell responsiveness remain controversial (13–15), it has been shown that different degrees of adaptive tolerance are induced in CD4 T cells chronically exposed to different amounts of nominal Ag (16). Thus, the mechanisms tuning activation thresholds in T cells may be quite diverse and remain ill defined.

To investigate whether mature T cells are endowed with adaptive mechanisms coping with excessive autoreactivity, we sought to increase the excitability of the TCR signaling machinery by using a previously characterized gain-of-function mutant of the protein tyrosine kinase Zap70 (17, 18). Zap70 is crucial for T cell activation being recruited upon TCR engagement to the CD3-ζ complex (19), where its catalytic activity is augmented by phosphorylation of Tyr493 by the Src-related protein tyrosine kinase Lck (20). Activated Zap70 phosphorylates the T cell adapter linker for activation of T cells (LAT) that connects to all major signaling...
pathways (21). We and others have reported that T cell activation and thymic differentiation also crucially depend on the phosphorylation of tyrosine Tyr319 in interdomain B of Zap70 (18, 22, 23). Our previous studies demonstrated that phosphorylated Tyr319 generates a site (pY319SDK motif) where Lck binds via its Src homology 2 domain, thus favoring Zap70 activation (17). Consistently, mutation of Y319SDK to Y319EEI, which augmented Lck-Src homology 2 binding affinity for Zap70 (24), leads to a gain-of-function of Zap70 (ZAP-YEEI), as detected by the strong increase in TCR-dependent signaling (17). Based on these data, we have engineered a transgenic (Tg) mouse model in which ZAP-YEEI is expressed in postselection CD8 single-positive (SP) thymocytes and mature CD8 T cells using the CD8-specific enhancer E8I (also named E8SP) (25, 26). This system allowed us to show that naive T cells and postselection SP thymocytes dynamically modify their signaling capacity when confronted with augmented levels of stimuli by spMHC. Evidence for signaling adaptation in mature T cells as a function of TCR-spMHC strength of interaction was obtained when these mice were crossed onto TCR-Tg mice P14 and HY, representing prototypes of high and low avidity for spMHC, respectively. Moreover, unexpected premature expression of Zap70-YEEI in double-positive (DP) thymocytes of P14 and HY TCR-Tg mice allowed us to demonstrate that modulation of TCR and CD5 expression can occur in response to augmented spMHC-derived stimuli during thymocyte maturation, possibly constituting a signaling adaptation mechanism integrating classic thymic selection schemes. Collectively, our data strongly suggest that CD8 thymocytes and T cells are able to reprogram their signaling machinery in reaction to TCR-received stimuli that reflect avidity for spMHC.

Materials and Methods

Animals

C57BL/6 mice were purchased from Charles River Laboratory. C57BL/6 Rag2−/− mice Tg for the P14 TCR (Vα2; Vβ8.1) or the anti-HY TCR (Vα3.70; Vβ8.2) and B6.CD3ε−/− mice obtained from the Centre de Distribution, Typage et Archivage Animal. HYRag2 mice used in all experiments were 3–6 wk of age. The Tg construct bearing the CD8-specific enhancer E8I (i.e., Tg-d in Ref. 25) and the minimal murine CD8 promoter was used to generate Zap70-YEEI mice (ZAP-YEEI-Tg). Tg mice were obtained when these mice were crossed onto TCR-Tg mice P14 and HY, representing prototypes of high and low avidity for spMHC, respectively. Moreover, unexpected premature expression of Zap70-YEEI in double-positive (DP) thymocytes of P14 and HY TCR-Tg mice allowed us to demonstrate that modulation of TCR and CD5 expression can occur in response to augmented spMHC-derived stimuli during thymocyte maturation, possibly constituting a signaling adaptation mechanism integrating classic thymic selection schemes. Collectively, our data strongly suggest that CD8 thymocytes and T cells are able to reprogram their signaling machinery in reaction to TCR-received stimuli that reflect avidity for spMHC.

Generation of Tg mice

The Tg construct bearing the CD8-specific enhancer E8I (i.e., Tg-d in Ref. 25) and the minimal murine CD8 promoter was used to generate Zap70-YEEI-Tg mice. The original hCD2 reporter gene was excised by Sall digestion of the plasmid and was replaced by the cDNA encoding for Zap70-YEEI (17). The S1 and S2 founders were generated at the Astra-Zeneca Transgenic Center and the Service d’Expérimentation Animale et Transgénèse, respectively, by injecting the NotI-digested expression cassette in fertilized C57BL/6 × CBA eggs. Injected ova were placed in pseudopregnant recipients. Offspring were genotyped by tail biopsy and Southern blot using the entire 1.9-kb Zap70-YEEI coding sequence as a probe. The Tg lines were maintained by repeated crossing to the C57BL/6 strains for 10 generations.

Abs and flow cytometry (FCM)

The following mAbs were used for flow cytometry: anti-CD8α-alkaline phosphocyanin (53-6-7), anti-TCR-β-alkaline phosphocyanin (H57-597), anti-Vo2a-biotin (B20.1), anti-CD4-PE (L3T4/RM4-5), anti-Cd69-FITC (H1.2F3), anti-CD25-FITC (7D4), anti-CD24/heat-stable Ag (HSA)-FITC or -biotin (M1/69), anti-CD44-FITC (IM781), and anti-CD62L-FITC (MEL14) from BD Pharmingen; anti-IFN-γ PE (2H7), anti-IL-4-PE (H129.19), anti-IL-10-PE (JES516), and anti-mouse IgG-APC (Amersham Biosciences Europe) and anti-hamster IgG and anti-rabbit-HRP (Jackson ImmunoResearch Laboratories).

CD8 T cells purification, stimulation, immunoprecipitation, and immunoblotting

Splenic CD8 T cells were purified by positive selection using CD8αε (Ly-2) MicroBeads and an AutoMACS magnetic cell sorter (Miltenyi Biotec), yielding cell suspensions containing 96–98% CD8 T cells. For immunoprecipitation and immunoblotting, CD8 T cells were stimulated by incubating them for 20 min on ice with 10 ng/ml anti-CD3 mAb (2C11) and subsequently cross-linking with 10 μg/ml anti-hamster IgG at 37°C for the indicated time. Cells were lysed at 4°C in 1% Nonidet P-40 and 1% TNF-α solvent (DNase) in complete RPMI 1640 medium (containing 10% FCS, 5 × 10−3 M 2-ME, 1 mM HEPES, and antibiotics). Cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 Abs or by adding the TCR-specific peptides gp33–41 and Smcy-3 (28) in the presence of 10 ng/ml of either anti-mouse H-2Db or H-2; Vα2-biotin (B20.1), anti-CD4-PE (L3T4/RM4-5), anti-CD69-FITC (28-14-8) or isotype-matched control Ab (G155-178; both from BD Pharmingen). At the indicated times, cells were stained with anti-CD8-PerCP (clone 53-6-7), anti-TCR-β-PE and anti-VSV-Alexa 488 and FCM. Alternatively, cells were stimulated with anti-CD3 and anti-CD28 Abs or by adding the TCR-specific peptides gp33–41 and Smcy-3 (28) in the presence of 10 ng/ml of either anti-mouse H-2Db or H-2; Vα2-biotin (B20.1), anti-CD4-PE (L3T4/RM4-5), anti-CD69-FITC (28-14-8) or isotype-matched control Ab (G155-178; both from BD Pharmingen). At the indicated times, cells were stained with anti-CD3 and anti-CD8-PE and anti-VSV-Alexa 488 and FCM.

Cytokine production

Spleen cells from C57BL/6 mice and TCR-Tg mice expressing Zap70-YEEI were incubated in 96-well plates (2.5 × 103 cells/well) at 37°C in 5% CO2 in complete RPMI 1640 medium (containing 10% FCS, 5 × 10−3 M 2-ME, 1 mM HEPES, and antibiotics). Cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 Abs or by adding the TCR-specific peptides gp33–41 and Smcy-3 (28) in the presence of 10 ng/ml anti-hamster IgG at 37°C for the indicated time. Cells were lysed at 4°C in 1% Nonidet P-40 and 1% TNF-α solvent (DNase) in complete RPMI 1640 medium (containing 10% FCS, 5 × 10−3 M 2-ME, 1 mM HEPES, and antibiotics). Cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 Abs or by adding the TCR-specific peptides gp33–41 and Smcy-3 (28) in the presence of 10 ng/ml of either anti-mouse H-2Db or H-2; Vα2-biotin (B20.1), anti-CD4-PE (L3T4/RM4-5), anti-CD69-FITC (28-14-8) or isotype-matched control Ab (G155-178; both from BD Pharmingen). At the indicated times, cells were stained with anti-CD3 and anti-CD8-PE and anti-VSV-Alexa 488 and FCM. Alternatively, cells were stimulated with anti-CD3 and anti-CD28 Abs or by adding the TCR-specific peptides gp33–41 and Smcy-3 (28) in the presence of 10 ng/ml of either anti-mouse H-2Db or H-2; Vα2-biotin (B20.1), anti-CD4-PE (L3T4/RM4-5), anti-CD69-FITC (28-14-8) or isotype-matched control Ab (G155-178; both from BD Pharmingen). At the indicated times, cells were stained with anti-CD3 and anti-CD8-PE and anti-VSV-Alexa 488 and FCM.

Peripheral T cell transfers

Nonirradiated male B6.CD3ε−/− hosts were injected i.v. with CD8 lymph node (LN) T cell populations from P14Rag2 mice or P14Rag2 breathing air. Donor T cells were purified by cytokine production after in vitro culture as described previously (18). Spleen cells from C57BL/6 mice expressing Zap70-YEEI were cultured in 96-well plates (2.5 × 105 cells/well) in complete RPMI 1640 medium supplemented with murine IL-7 (1/100 dilution of a supernatant from J558 cells transfected with an IL-7 CANA; a gift from F. Melchers, University of Basel, Basel, Switzerland) (29) and 10 μg/ml of either anti-mouse H-2Db (28-14-8) or isotype-matched control Ab (G155-178; both from BD Pharmingen). At the indicated times, cells were stained with anti-CD8-PE and anti-VSV-Alexa 488 and FCM.

Results

Generation of Tg mice expressing a Zap70 gain-of-function mutant in mature CD8 thymocytes and peripheral CD8 T cells

Mouse oocytes were injected with a construct containing the CD8αε gene promoter flanked by the E8I enhancer (25), driving expression of the gain-of-function mutant of Zap70, ZAP-YEEI, with a C-terminal-terminated VSV epitope tag (17) (Fig. 1A). Normally, E8I directs gene expression only in postselected CD8 SP
FIGURE 1. ZAP-YEEI expression in thymocytes and peripheral CD8 T lymphocytes from transgenic mice. A, Schematic representation of the transgenic construct (modified from Ref. 25). P8a, minimal CDSa promoter; CD4, splicing module composed of the untranslated exon I, part of intron I, and part of exon II from the murine CD4 gene; pA, SV40 polyadenylation site. B, Lymph node cells from SZ1 (left panel) and SZ2 (right panel)-Tg mice, were surface stained with anti-CD8 Abs, then fixed, permeabilized, and stained with the anti-VSV Ab to detect ZAP-YEEI. The percentage of ZAP-YEEI-positive cells in the CD8 population from ZAP-YEEI Tg mice (black line) is indicated within each panel. C, Anti-VSV staining of control cells from wild-type littermates. Thymocytes were analyzed by FCM after staining with anti-CD8-allophycocyanin, anti-CD4-PE, anti-HSA-PerCP, and anti-VSV-Alexa488. CD4 vs CD8 dot plots are displayed for total thymocytes (left panels). Histograms show the expression of ZAP-YEEI (middle panels) and HSA (right panels) in ZAP-YEEI Tg mice (black line) and wt mice (gray) in the thymocyte population gated in the corresponding left panel.

ZAP-YEEI expression did not alter the frequency of thymic subsets or the expression of TCR, CD4, CD8, CD5, CD28, CD69, CD44, CD45, CD25, and CD62L comparable to those found in C57BL/6 wt littermates and did not up-regulate CTLA4 (data not shown). Notably, however, ZAP-YEEI+CD8 T cells from both SZ1 and SZ2 LN showed decreased TCR amounts (~50% reduction in mean fluorescence intensity (MFI) of TCRβ; Fig. 2A, middle and right panels). A similar decrease in TCR expression was observed in ZAP-YEEI+CD8 T cells from spleen (not shown) and ZAP-YEEI+CD8 SP thymocytes (Fig. 2A, left panel). It is unlikely that lower TCR levels were consequent to deletion of TCRβhigh thymocytes, because negative selection was not noticeably augmented in these animals (see above). The TCR may be down-regulated as a consequence of ligand binding, followed by internalization and degradation (31). Therefore, it is likely that stronger signaling after interactions with spMHIC may induce TCR down-regulation in ZAP-YEEI+ cells without inducing activation.

Spleen and LN CD8 T cells from ZAP-YEEI-Tg mice displayed an apparent naive phenotype, because they expressed levels of CD8, CD28, CD69, CD44, CD45, CD25, and CD62L comparable to those found in C57BL/6 wt littermates and did not up-regulate CTLA4 (data not shown). Notably, however, ZAP-YEEI+CD8 T cells from both SZ1 and SZ2 LN showed decreased TCR amounts (~50% reduction in mean fluorescence intensity (MFI) of TCRβ; Fig. 2A, middle and right panels). A similar decrease in TCR expression was observed in ZAP-YEEI+CD8 T cells from spleen (not shown) and ZAP-YEEI+CD8 SP thymocytes (Fig. 2A, left panel). It is unlikely that lower TCR levels were consequent to deletion of TCRβhigh thymocytes, because negative selection was not noticeably augmented in these animals (see above). The TCR may be down-regulated as a consequence of ligand binding, followed by internalization and degradation (31). Therefore, it is likely that stronger signaling after interactions with spMHIC may induce TCR down-regulation in ZAP-YEEI+ cells without inducing activation.

CD8 T cells from ZAP-YEEI-Tg mice also displayed a slight, but reproducible, reduction in CD5 expression (Fig. 2B). CD5 attenuates TCR signaling (9), and its expression level positively correlates with the strength of TCR-induced signals (10, 13). Although the reason for the decrease in CD5 expression in this setting was unclear (see Discussion), these data suggested that persistent augmentation of spMHIC-generated TCR signals in vivo modulates both TCR and CD5 expression in mature T cells.

Reduced cytokine production by CD8 T cells expressing ZAP-YEEI

These results prompted examination of the functional response of CD8 T cells expressing ZAP-YEEI to TCR and CD28 costimulation. As shown in Fig. 3A, IFN-γ production, as detected by intracellular staining, was appreciably reduced in the ZAP-YEEI+ DP thymocytes, but it was expressed in CD8 SP cells (Fig. 1C). The lower frequency of cells expressing ZAP-YEEI in CD8 SP thymocytes compared with LN CD8 T cells (i.e., 50 vs 75%) was probably due to maturation-dependent increased expression from the E8 enhancer (25). Consistently, the amount of ZAP-YEEI in CD8 SP thymocytes augmented with HSA down-regulation (Fig. 1C), coincident with completion of positive selection (30). ZAP-YEEI expression did not alter the frequency of any thymic subset (percentages for Tg vs wt mice, respectively, were 6.7 ± 0.2 vs 5.1 ± 1.3 for DN, 80.4 ± 1.4 vs 81.8 ± 3.6 for DP, 9.1 ± 2.4 vs 9.6 ± 3.7 for SP CD4, and 3.8 ± 0.9 vs 3.6 ± 1.1 for SP CD8; n = 3 for all data), in particular, the percentage of mature HSAlow CD8 SP thymocytes (Fig. 1C), indicating no overt effect on thymic selection processes. Spleen and LN from ZAP-YEEI-Tg mice also had normal cellularity (not shown).

FIGURE 2. TCR and CD5 expression in ZAP-YEEI-expressing CD8 T lymphocytes. FCM analysis of cells stained with anti-CD8-allophycocyanin, anti-CD4-PE, and anti-TCRβ-PerCP and intracellularly stained with anti-VSV-Alexa488. A, ZAP-YEEI vs TCRβ profiles are displayed for CD8 SP thymocytes and CD8 T lymph node cells from SZ1 and SZ2 mice. The percentage of transgene-positive and transgene-negative cells is indicated. B, Expression of CD5 on peripheral CD8 T cells from ZAP-YEEI Tg (black line) and control mice (gray).
comparing with the ZAP-YEEI− subset. Similar results were obtained for TNF-α and IL-2 production (Fig. 3C). Both the frequency of cytokine-producing cells and the amount of cytokine produced per cell were lower (Fig. 3, A and C). Lower IFN-γ and IL-2 secretion by CD8 T cells from SZ1 mice was confirmed by ELISA (not shown). However, cells stimulated with PMA and a Ca2+ ionophore produced equal levels of IFN-γ (Fig. 3B) and other cytokines (data not shown) regardless of ZAP-YEEI expression. Thus, expression of ZAP-YEEI decreased activation-induced cytokine production, a defect apparently caused by attenuation of TCR-proximal signaling.

Modified TCR-proximal signaling in CD8 T cells from ZAP-YEEI-Tg mice

To investigate alterations in TCR-proximal biochemical events that might explain the reduced activation of ZAP-YEEI− cells, the phosphorylation status of Zap70 in splenic CD8 T cells from normal C57BL/6 and SZ1 mice was examined. Zap70 tyrosine phosphorylation in unstimulated control cells (Zap70wt) was hardly detectable, but underwent a sharp increase upon anti-CD3 cross-linking (Fig. 4A). In contrast, phosphorylation of ZAP-YEEI (migrating with reduced mobility because of the appended epitope tag; Fig. 4A, lower panel) was detected even in unstimulated cells, and its intensity increased upon anti-CD3 stimulation well above the levels seen in endogenous Zap70wt cells (Fig. 4A, upper panel). As previously demonstrated in Jurkat cells (17), the higher phosphorylation of ZAP-YEEI compared with Zap70wt correlates with the higher TCR-induced kinase activity of the former. Consistently, unstimulated cells from SZ1 mice had increased basal phosphorylation of the 36-kDa transmembrane adaptor LAT (3- to 4-fold higher than in normal mice; Fig. 4, B and C), a major Zap70 substrate (21). The hyperphosphorylation of ZAP-YEEI and LAT was not induced by CD8 cross-linking during the positive selection procedure we routinely used to purify CD8 T cells, because identical results were obtained with cells isolated by negative selection (not shown). The magnitude of gain-of-function of ZAP-YEEI previously measured in Jurkat cells (e.g., 20- to 30-fold greater capacity to stimulate NF-AT activation compared with Zap70wt) (17) makes it unlikely that changes in basal and induced phosphorylation were due to the mere 2- to 3-fold increase in total Zap70 (endogenous Zap70 plus ZAP-YEEI), although we cannot...
rule out a contribution due to such a limited protein overexpression. Thus, although these data agree with the prediction that ZAP-YEEI confers higher TCR-proximal signaling, its expression in peripheral T cells reduced T cell activation. One explanation may reside in reduced TCR levels and/or other negative feedback mechanisms compensating for augmented Zap70 function as part of an adaptive behavior. Indeed, comparison of the kinetics of anti-CD3-induced phosphorylation in CD8 T cells from SZ1 and control mice revealed that although after 30-s stimulation with anti-CD3, LAT phosphorylation was slightly higher in the former, at later time points this difference disappeared (i.e., 2 min; Fig. 4B) and then reversed (i.e., 5 and 10 min; Fig. 4, B and C). ERK2 also underwent faster dephosphorylation kinetics in SZ1 compared with normal mice (Fig. 4B, lower panels). Collectively, these data indicate that expression of ZAP-YEEI results in higher basal and early CD3-induced LAT phosphorylation. However, the activation signal is down-regulated more quickly, providing a potential explanation for the reduced TCR-induced cytokine production, which requires sustained signaling (32).

Recovery of TCR expression and cytokine production in ZAP-YEEI-expressing CD8 T cells upon self-stimuli deprivation

To directly address whether the reduction of TCR levels and cytokine production in ZAP-YEEI-expressing cells was the result of dynamic signal tuning upon increased self-stimulation, we cultured purified CD8 T cells from SZ1 mice in the presence of an anti-H-2Db Ab to prevent TCR interaction with spMHC. IL-7 was also added to sustain naive T cell survival in vitro (33). TCR expression by ZAP-YEEI+ cells significantly increased within the first 24–48 h. Consequently, the ratio between TCRβ MFI on ZAP-YEEI+ and ZAP-YEEI- CD8 T cells changed from 2 to 1.2 and remained stable for an additional 48 h (Fig. 5A). The recovery of TCR expression in ZAP-YEEI+ cells was accompanied by a functional resetting of the signaling machinery. Indeed, when we measured CD3/CD28-induced IFN-γ production by cells cultured for 72 h under the same conditions, we found that ZAP-YEEI+ cells produced the same or even higher levels of IFN-γ as ZAP-YEEI- cells (20.3 ± 4.7 vs 15.9 ± 3.6; n = 4; Fig. 5B). These data indicated that reduced TCR expression and activation in ZAP-YEEI-expressing cells required continuous interaction of the TCR with spMHC.

ZAP-YEEI in TCR-Tg background mice: unpredicted expression in DP thymocytes and inverse correlation between TCR levels and avidity for spMHC

The above data showed that TCR-dependent signals induce a phenotypic and functional tuning of mature T cells. One prediction would then be that TCR avidity for spMHC and negative tuning of the signaling machinery are directly correlated and that T cells with the highest down-modulated TCR within the ZAP-YEEI+ cells (Fig. 2A) would be those bearing TCR with higher affinity/avidity for spMHC. To directly test these ideas, SZ1-Tg mice were crossed onto mouse lines expressing the P14 and HY αβ TCR transgenes (34, 35). Both these receptors are positively selected by H-2Db, but P14 displays a higher avidity for spMHC than HY (10, 11, 36). However, one unexpected finding apparently complicated the study in these dTg mice in that, in contrast to C57BL/6 mice (Fig. 1), ZAP-YEEI expression started at the DP stage on both TCR-Tg backgrounds (Fig. 6, A and B). The reason for the earlier activation of the E8I enhancer is unclear, but it is probably due to premature expression and signaling of the transgenic αβ TCR in developing thymocytes (28, 37). Hence, in these models, ZAP-YEEI might modify signaling during thymocyte maturation, thus affecting positive and/or negative selection processes.

FACS analysis of P14 × ZAP-YEEI dTg thymi revealed ZAP-YEEI expression in 40% of DP cells, increasing up to 78% in the CD8 SP subset (Fig. 6A, middle panels). Similar to SZ1 and SZ2 mice (see Fig. 2), there was an inverse correlation between the expression of ZAP-YEEI and the P14 TCR (Fig. 6A). In particular, the Vα2high population, representing cells that up-regulated the TCR level after positive selection (30), was strongly reduced in CD8 SP cells and in the CD8αlowβhighCD8βhigh transitional population from P14 × ZAP-YEEI thymi (Fig. 6A, right panels in the last two rows). A weak TCR down-regulation was also observed in DP cells (note the slight drift to the right and the increased cell number of the DP Vα2 lower expressors; Fig. 6A). The expression of CD5 was also weakly reduced in CD8 SP cells (Fig. 6C). Although reduction of TCRhigh SP thymocytes is normally interpreted as a sign of increased negative selection, this did not appear to be the case, because we found that the fraction of mature CD8 SP thymocytes with down-regulated HSA was similar in P14 and P14 × ZAP-YEEI (Fig. 6C), and no significant differences in the absolute numbers of CD8 SP thymocytes were detected (Table I). These data are consistent with maturing P14 thymocytes reacting to a lower activation threshold conferred by ZAP-YEEI by tuning TCR levels, hence limiting/avoiding negative selection.

In HY × ZAP-YEEI female mouse thymi, the expression of ZAP-YEEI also began at the DP stage (~25% of ZAP-YEEI+ cells; Fig. 6B) and gradually increased with maturation, reaching almost 95% in the CD8 SP subset (Fig. 6B). However, in contrast to P14 × ZAP-YEEI mice, HY TCR was not down-regulated. Indeed, T3.70high cells appeared earlier in HY × ZAP-YEEI than in control HY mice, concomitant with reduced DP TCRlow cells (Fig. 6B, right panel in the first row) and were of comparable frequency in mature CD8 SP thymocytes (Fig. 6B, right panel in the bottom row). Moreover, the percentage of HSAlow cells (Fig. 6C) and the absolute number of CD8 SP thymocytes (Table I) were

**FIGURE 5.** TCR expression and cytokine production after in vitro culture of ZAP-YEEI-expressing T cells. Purified CD8 T cells (2.5 × 10^6) from ZAP-YEEI-Tg mice were incubated with anti-H-2Db Abs in the presence of IL-7. A. At the indicated time, cells were triple stained for CD8, TCRβ, and ZAP-YEEI. Each point represents the ratio of TCRβ MFI from ZAP-YEEI-negative and ZAP-YEEI-positive cells (mean ± SD; n = 4 mice). B. Cells were stimulated with anti-CD3 and anti-CD28 Abs immediately (right panel) or after 72 h of culture with anti-H-2DbAb (left panel). Cells were collected 20 h later and stained with anti-CD8, anti-IFN-γ, and anti-VSV Abs. IFN-γ was measured in CD8-gated ZAP-YEEI-negative and ZAP-YEEI-positive cells. For each population, the percentage of cytokine-producing cells is indicated. Similar results were obtained in four other independent experiments.
CD8 T cells (90–95%) expressed the mutant. No changes in the expression levels of CD25, CD44, CD62L, and CD69 were evident in unstimulated cells (data not shown). However, TCR expression was differently affected in these mice. Vα2 Tg TCR in CD8 T cells of P14 × ZAP-YEEI mice was ~3.5-fold lower than that in control P14 littermates (Fig. 7, middle row, left panel). A smaller reduction (1.7-fold) in clonotypic T3.70 TCR expression was also seen in HY × ZAP-YEEI females compared with HY littermates (Fig. 7, middle right panel). The apparent decrease in either Vα2 or T3.70 staining was not due to the expression of a second TCR α-chain induced by stronger signaling, because a similar reduction in clonotypic TCRs was observed on a Rag2−/− background, where the expression of alternative transcripts is suppressed (data not shown). Similar to SZ1 mice (Fig. 2), CD5 was slightly decreased in P14 × ZAP-YEEI CD8 T cells compared with P14 littermates (Fig. 7, bottom left panel). Surprisingly, CD5 in CD8 T cells from female HY × ZAP-YEEI mice was significantly augmented compared with that in control cells (Fig. 7, bottom right panel). Based on the proposed correlation between CD5 levels and the strength of TCR-mediated signaling (10, 13), these results suggest that TCR-dependent signals are reduced in P14 × ZAP-YEEI T cells, although they are increased in HY × ZAP-YEEI T cells. Accordingly, we found that the number of LN CD8 T cells in P14 × ZAP-YEEI was reduced compared with that in P14 mice, whereas it was augmented in HY × ZAP-YEEI mice compared with that in HY littermates (Table I). Additional confirmation of the reduced response to spMHC-induced stimuli of P14 × ZAP-YEEI cells was obtained by transferring these cells into lymphopenic hosts. Equal numbers of CD8 T cells purified from P14Rag2−/− × ZAP-YEEI and P14Rag2−/− animals were mixed and transferred into CD3ε−/− syngenic hosts. Spleens were removed at different times after transfer, and the absolute number and percentage of P14Rag2−/− × ZAP-YEEI- and P14Rag2−/−-derived cells were assessed by intracellular staining with anti-VSV Ab. Although the number of P14Rag2−/−-derived cells significantly increased after transfer, a much lower increase was observed for P14Rag2−/− × ZAP-YEEI cells (Fig. 8A), demonstrating that the ability of the latter to undergo lymphopenia-driven expansion was impaired. Interestingly, we observed a slight reduction in IL-7R α-chain levels on P14 cells expressing ZAP-YEEI compared with normal P14 T cells (Fig. 8B). Although the significance of this finding is still unclear, IL-7 has been proposed as a major regulator of both survival of naïve T cells in normal mice and their expansion in lymphopenic hosts (reviewed in Ref. 33). Thus, decreased IL-7Rα levels may contribute to the defects observed in P14 × ZAP-YEEI cells.

Overall, the expression of ZAP-YEEI in P14- and HY TCR-bearing cells suggests that signal threshold tuning is initiated during thymocyte development and could continue in the peripheral compartment, as suggested by TCR and CD5 level modulation.

Contrasting functional responses in vitro of P14 and HY T cells expressing ZAP-YEEI

The consequences of the divergent adaptation of CD8 T cells from dTg mice on the induction of effector functions were assessed by analyzing their response to Ag stimulation in vitro. Splenocytes from P14 and P14 × ZAP-YEEI mice or from female HY and HY × ZAP-YEEI mice were cultured in the presence of specific agonist peptides for 20 h, and cytokine production by CD8 T cells was determined by intracellular staining and flow cytometry. The percentage of cells producing IFN-γ, IL-2, and TNF-α in response to saturating concentrations of the antigenic peptide was lower in P14 × ZAP-YEEI splenocytes compared with control cells (Fig. 9A), a result reminiscent of that obtained for ZAP-YEEI-expressing T cells from C57BL/6 mice. In contrast to the P14 model, the

not different from those in HY female littermates. These data indicate that the expression of ZAP-YEEI in DP thymocytes does not grossly alter their development. Rather, thymocytes appear to cope with signal excess in a different way, probably dependent on their high and low avidities for their selecting ligand(s), respectively: down-regulating TCR levels and limiting negative selection for P14 or accelerating maturation for HY.

Divergent consequences of ZAP-YEEI expression on homeostasis of peripheral CD8 T cells in P14 and HY transgenic mice

Similar to SZ1 and SZ2 mice, peripheral T cells from P14 × ZAP-YEEI and female HY × ZAP-YEEI mice expressed higher levels of ZAP-YEEI than thymocytes (Fig. 7, top panels), and almost all CD8 T cells (90–95%) expressed the mutant. No changes in the
fraction of cytokine-producing CD8 T cells in HY × ZAP-YEEI mice was increased compared with control HY CD8 cells (Fig. 9B). Thus, despite the decrease in TCR levels and the increase in the negative regulator CD5 on HY × ZAP-YEEI CD8 T cells, their responsiveness to Ag stimulation is improved by ZAP-YEEI expression.

Discussion

Proposed more than a decade ago on theoretical grounds, TCR signal tuning is conceived as a cell-intrinsnic device integrating selection-based mechanisms that maintain self-recognition and tolerance (7). However, when and how such tuning is achieved are unclear. During thymocyte maturation, spMHC-generated signals appear to trigger adjustments in expression levels of surface receptors, including CD5 and CD2, which, in turn, would regulate TCR sensitivity (10, 11, 38). Although CD5 may play a similar role in setting the activation threshold of peripheral naive T cells (11–13), it is not known whether other receptors and/or signaling proteins are involved in such a regulation. Moreover, modifications of T cell responsiveness resembling signal tuning have been observed in CD4 T cells stimulated by chronic exposure to Ag (16).

In this study we tested the signaling tuning capacity of naive T cell challenged in vivo with increased excitation by self-stimuli. This condition was achieved by targeting the expression of a gain-of-function Zap70 mutant at the onset of CD8 SP thymocyte appearance and in mature CD8 T cells. Our data suggest that in mice with a normal TCR repertoire, most naive CD8 T cells tune their signaling machinery toward a higher activation threshold when experiencing stronger and prolonged excitation after spMHC interaction, a scenario predicted by the tunable activation threshold hypothesis (7). Consistent with signaling adaptation, ZAP-YEEI-expressing CD8 T cells showed no sign of activation in vivo, but, on the contrary, displayed a lower TCR signaling status, as demonstrated by reduced in vitro cytokine secretion in response to TCR/CD28 costimulation. Both TCR expression and responsiveness to stimulation were dynamically tuned at the single-cell level and could be restored when cells were deprived of TCR/spMHC interaction for several hours.

The apparently contradictory observation of a higher in vivo excitation to spMHC, detected as basal LAT phosphorylation (Fig. 8A). Approximately 5 × 10⁸ LN cells from P14 Rag-Tg (black line) and P14 Rag-YEEI (black line) mice were cotransferred into B6.CD3 /H11005 mice. The distinction between the two mouse populations was conducted through intracellular staining with the anti-VSV Ab. Each point in the graph represents the total number of CD8 cells recovered from the spleens of the hosts (mean ± SD of three mice per time point).

**FIGURE 8.** Proliferation of P14 CD8 T cells expressing ZAP-YEEI in T lymphocyte-deficient hosts and expression IL7Rα chain in P14 × ZAP-YEEI mice. A. Approximately 5 × 10⁸ LN cells from P14Rag-/- (□) and P14Rag-/- × ZAP-YEEI (■) mice were cotransferred into B6.CD3e hosts. The distinction between the two mouse populations was conducted through intracellular staining with the anti-VSV Ab. Each point in the graph represents the total number of CD8 cells recovered from the spleens of the hosts (mean ± SD of three mice per time point). B. FCM analysis of cells stained with biotinylated anti-Va2 Ab-streptavidin-PerCP, anti-CD8-allophycocyanin, and anti-CD5-PE and intracellularly stained with anti-VSV-Alexa488. Histograms show the expression of ZAP-YEEI, clonotypic TCR and CD5 on TCR-Tg (black line) and TCR-Tg (open line) by gated CD8 T cells. The percentage of transgene-expressing cells and the ratio of MFI of clonotypic TCR and CD5 of TCR-Tg vs that of TCR-Tg × ZAP-YEEI mice are indicated.

**FIGURE 7.** Phenotypic characterization of peripheral CD8 T cells of P14 and H-Y mice expressing ZAP-YEEI. FCM analysis of cells stained with anti-clonotypic Abs conjugated to biotin-streptavidin-PerCP, anti-CD8-allophycocyanin, and anti-CD5-PE and intracellularly stained with anti-VSV-Alexa488. Histograms show the expression of ZAP-YEEI, clonotypic TCR and CD5 on TCR-Tg × ZAP-YEEI (black line) and TCR-Tg mice (open line) by gated CD8 T cells. The percentage of transgene-expressing cells and the ratio of MFI of clonotypic TCR and CD5 of TCR-Tg vs that of TCR-Tg × ZAP-YEEI mice are indicated.

### Table I. Absolute numbers of CD8 T cells in TCR-Tg and dTg mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Va2⁺CD8⁺</th>
<th>T3.70⁺CD8⁺</th>
<th>P14 CD8-SP</th>
<th>HY CD8-SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus</td>
<td>ZAP-WT</td>
<td>ND</td>
<td>ND</td>
<td>17.62 ± 9.14 (n = 9)</td>
</tr>
<tr>
<td></td>
<td>ZAP-YEEI</td>
<td>ND</td>
<td>ND</td>
<td>14.11 ± 6.05 (n = 9)</td>
</tr>
<tr>
<td>LN</td>
<td>ZAP-WT</td>
<td>9.18 ± 1.22 (n = 3)</td>
<td>5.43 ± 2.37 (n = 4)</td>
<td>8.44 ± 3.05 (n = 4)</td>
</tr>
<tr>
<td></td>
<td>ZAP-YEEI</td>
<td>5.79 ± 1.33 (n = 3)</td>
<td>15.77 ± 6.06 (n = 4)</td>
<td>8.04 ± 3.05 (n = 4)</td>
</tr>
</tbody>
</table>

a Total thymocytes and LN cells from control (ZAP-WT) or dTg (ZAP-YEEI) mice were isolated and counted. The number of clonotypic TCR-positive, CD8-SP cells or of CD8-SP cells in each sample was calculated after assessing the percentage of these cells by FCM analysis.

b These data were obtained by counting cells after pooling samples and dividing by indicated numbers of mice. Data represent mean ± SE (number × 10⁶).

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**DISCUSSION**

Proposed more than a decade ago on theoretical grounds, TCR signal tuning is conceived as a cell-intrinsic device integrating selection-based mechanisms that maintain self-recognition and tolerance (7). However, when and how such tuning is achieved are unclear. During thymocyte maturation, spMHC-generated signals appear to trigger adjustments in expression levels of surface receptors, including CD5 and CD2, which, in turn, would regulate TCR sensitivity (10, 11, 38). Although CD5 may play a similar role in setting the activation threshold of peripheral naive T cells (11–13), it is not known whether other receptors and/or signaling proteins are involved in such a regulation. Moreover, modifications of T cell responsiveness resembling signal tuning have been observed in CD4 T cells stimulated by chronic exposure to Ag (16).

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The apparently contradictory observation of a higher in vivo excitation to spMHC, detected as basal LAT phosphorylation (Fig. 8A). Approximately 5 × 10⁸ LN cells from P14 Rag-Tg (black line) and P14 Rag-YEEI (black line) mice were cotransferred into B6.CD3e hosts. The distinction between the two mouse populations was conducted through intracellular staining with the anti-VSV Ab. Each point in the graph represents the total number of CD8 cells recovered from the spleens of the hosts (mean ± SD of three mice per time point). B. FCM analysis of cells stained with biotinylated anti-Va2 Ab-streptavidin-PerCP, anti-CD8-allophycocyanin, and anti-CD5-PE and intracellularly stained with anti-VSV-Alexa488. Histograms show the expression of ZAP-YEEI, clonotypic TCR and CD5 on TCR-Tg (black line) and TCR-Tg mice (open line) by gated CD8 T cells. The percentage of transgene-expressing cells and the ratio of MFI of clonotypic TCR and CD5 of TCR-Tg vs that of TCR-Tg × ZAP-YEEI mice are indicated.
A TCR-specific peptide or the HY TCR-specific peptide (5–10^6 HY-ZAP-YEEI CD8 T cells. Splenocytes from P14 (B6) and HY (B10) mice were stimulated in culture for 20 h with the gp33–41 P14 TCR-specific peptide or the HY TCR-specific peptide (5 × 10^6 M), respectively. Cells (1 × 10^6) were then stained with anti-CD8-allophycocyanin and anti-clonotypic-TCR conjugated to biotin-streptavidin-PerCP, fixed, permeabilized, and stained for anti-cytokine-PE Abs. Histograms show the production of IFN-γ, IL-2, and TNF-α of TCR-Tg (□) or TCR-Tg × ZAP-YEEI (■) by gated CD8^+ clonotypic TCR^+ cells. A representative experiment of four is shown.

FIGURE 9. Ag-induced cytokine production by P14 × ZAP-YEEI and HY-ZAP-YEEI CD8 T cells. Splenocytes from P14 (A) and HY (B) mice expressing ZAP-YEEI were stimulated in culture for 20 h with the gp33–41 P14 TCR-specific peptide or the HY TCR-specific peptide (5 × 10^6 M), respectively. Cells (1 × 10^6) were then stained with anti-CD8-allophycocyanin and anti-clonotypic-TCR conjugated to biotin-streptavidin-PerCP, fixed, permeabilized, and stained for anti-cytokine-PE Abs. Histograms show the production of IFN-γ, IL-2, and TNF-α of TCR-Tg (□) or TCR-Tg × ZAP-YEEI (■) by gated CD8^+ clonotypic TCR^+ cells. A representative experiment of four is shown.

4), and a lower in vitro responsiveness may be explained in several ways. Cells on the brink of completing their TCR signal tuning (e.g., recent thymic emigrants) and/or cells bearing TCRs with low avidity for spMHC and improving their fitness by exploiting ZAP-YEEI may both reveal a stronger spMHC-driven basal signal and coexist with a majority of ZAP-YEEI-expressing cells having already tuned down their TCR signaling (lower responders). An alternative explanation is offered by the observation that ZAP-YEEI-expressing cells showed faster LAT and ERK-2 dephosphorylation after TCR triggering (Fig. 4, B and C). Thus, augmented early phosphorylation might occur in all ZAP-YEEI-expressing cells, but full activation could not take place because of fast signal dampening due to reduced TCR availability and/or increase in other negative feedback mechanisms (see below).

Studies of P14 and HY TCR-Tg mice expressing ZAP-YEEI supported the view that TCR signal tuning is largely dictated by TCR avidity for self-ligands. Thus, although expression levels of ZAP-YEEI protein in SP CD8 thymocytes of HY × ZAP-YEEI and P14 × ZAP-YEEI were comparable (compare Fig. 6, A and B), only the latter showed decreased TCR expression, correlating with TCR avidity for spMHC. Moreover, HY × ZAP-YEEI developing thymocytes displayed accelerated expression of TCR^high cells (Fig. 6B), and their mature counterpart accumulates in the periphery more than HY cells, just the opposite of the behavior of P14 × ZAP-YEEI thymocytes and mature CD8^+ T cells.

The contrasting behavior of mature T cells induced by a similar modification of their TCR signaling machinery is striking, but suggests that, depending on the initial strength of the TCR signal, the increased reactivity induced by ZAP-YEEI may be over- or undercompensated. Thus, the strong signal generated by ZAP-YEEI downstream of a high avidity or promiscuous TCR, such as P14, would induce a negative feedback in thymocytes and/or peripheral T cells, dampening their proliferation potential and limiting possible activation by self-ligand(s). On the contrary, the weak signal generated by a low avidity TCR such as HY, although reinforced by ZAP-YEEI, would still be compatible with positive selection and normal survival/expansion in the periphery. Hence, although some limited adaptation is induced through TCR and CD5 modulation, cells may acquire improved functional capacities.

Surprisingly, not all activation responses appeared to be equally affected by signal tuning in ZAP-YEEI-expressing T cells. For example, we never detected alterations in activation-induced up-regulation of CD69 or CD25 on polyclonal or Tg TCR backgrounds (data not shown). Moreover, proliferation did not seem to be affected in either S21 or P14 × ZAP-YEEI mice, whereas HY × ZAP-YEEI CD8^+ T cells proliferated slightly faster than HY T cells, as assessed by CSFE labeling (not shown). These unpredicted differences may be due to different activation thresholds of these responses compared with cytokine production or to the different sensitivities of the in vitro assays used.

The unanticipated expression in TCR-Tg mice of ZAP-YEEI in DP thymocyte provided a model for monitoring the fate of immature thymocytes subjected to more intense spMHC-induced signaling. Indeed, because Zap70 plays a pivotal role in both positive and negative thymic selection (39), ZAP-YEEI expression in DP may affect either event, or both, depending on TCR avidity for the selecting ligand(s). Because we did not detect significant differences in thymic cellularity, subpopulation composition, or HSA down-regulation, we argue that no gross alteration of positive and negative selection was taking place. However, P14 and HY TCR-bearing thymocytes were differently affected by ZAP-YEEI expression. On the one hand, TCR^low DP cells in P14 × ZAP-YEEI mice were slightly increased, and SP TCR^high cells were strongly decreased, suggesting that modulation of TCR levels may avoid negative selection when receiving strong spMHC stimuli. Similarly, a dramatic reduction of TCR levels in DP and TCR^high SP thymocytes was observed in P14 × CD5-deficient mice (11). Moreover, TCR^low mature T cells appeared in the periphery of these animals (11). However, DP and SP cells were decreased in these mice, indicating that the absence of CD5 leads to higher TCR signaling to spMHC than the expression of ZAP-YEEI. Hence, the latter situation set the thymocytes in a state compatible with a decrease in TCR expression rather than negative selection. In contrast, ZAP-YEEI expression in HY DP cells induced an earlier appearance of TCR^high cells at the DP and intermediate DP to SP stages. However, this effect did not increase mature thymocyte numbers compared with HY mice (compare HY and HY × ZAP-YEEI mice in Table I). Taken together, our data reveal that signaling plasticity based on TCR level modulation operates concomitantly with selection processes. Accordingly, it has been recently reported that surface levels of HY, 2C, and OT1 TCR-Tg cells inversely correlate with increasing avidity for MHC class I (40).

Our observations suggest that TCR level modifications in response to augmented signaling may take place during all T cell developmental stages, because a significant TCR reduction was evident in peripheral T cells. Indeed, TCR down-modulation in HY × ZAP-YEEI mice was detected only in mature T cells. Moreover, augmented TCR down-regulation was apparent in P14 × ZAP-YEEI T cells compared with SP thymocytes (from 2–3.5 times reduction compared with P14) despite similar ZAP-YEEI protein expression levels. Although the mechanism leading to TCR down-modulation in ZAP-YEEI-expressing cells is still unclear, we found that the expression of at least one of the TCR complex subunits, namely the TCR ζ-chain, was decreased only at the protein, not the mRNA, level (unpublished observations), indicating a post-translational regulatory mechanism possibly involving protein degradation.

If stronger signaling upon spMHC contact brings about TCR down-modulation in CD8^+ T cells, it can be predicted that reducing...
or eliminating spMHC-induced signals would result in the recovery of TCR levels. We show in this study that this was actually the case in ZAP-YEEI cells from SZ1 mice (Fig. 5), and that TCR-mediated responses were also restored under these conditions. A partial recovery of TCR expression was also observed in vivo upon transfer of P14Rag1-/- and P14Rag1-/- × ZAP-YEEI mice in CD3e-/- Dclh2-/- hosts. The initial 3.5-fold increased ratio in Va2 TCR MF1 between control and dTg cells was decreased to 2-fold 48 h after transfer (not shown). The incomplete recovery in this setting may be due to several reasons: for instance, the presence of MHC molecules on LN cells of donor origin or the expression of other MHC haplotypes in Dclh2-/- hosts may limit TCR recovery by P14 × ZAP-YEEI cells. Taken together, our results demonstrate that TCR expression and T cell responsiveness may be dynamically regulated in peripheral T cells according to the strength of continuous spMHC stimulation.

It is worth noting that despite similar or only slightly different TCR levels in CD8 T cells from C57Bl/6, P14, and HY T cells expressing ZAP-YEEI (2-, 3.5-, and 1.7-fold, respectively), responsiveness was decreased in the former two, but enhanced in the latter, suggesting that the TCR level is only one element contributing to hyporesponsiveness. Even though we attempted to identify modifications of known positive or negative signaling regulators, we found no changes in the expression levels of LAT, Lck, Fyn, Src homology region 2 domain-containing phosphatase-1, and c-Cbl (Fig. 4C and data not shown) in cells expressing ZAP-YEEI. Nonetheless, the faster dephosphorylation of LAT and ERK-2 occurring in these cells after TCR cross-linking suggests increased recruitment/activation of negative regulators (e.g., the protein tyrosine phosphatase Src homology region 2 domain-containing phosphatase-1) (41).

Another hallmark of TCR signal tuning is the modulation of CD5 levels (11, 13). We observed that CD5 levels were reduced in T cells manifesting lower responses in vitro and in vivo. In contrast, CD5 increased in T cells with improved responsiveness (e.g., HY × ZAP-YEEI T cells). Based on the negative regulation exerted by CD5 on TCR signaling (9), one would expect that an increase in CD5 expression would decrease T cell reactivity and vice versa. However, several observations have previously suggested that CD5 levels actually mirror the intensity of TCR-dependent signals (11, 13). Therefore, our results, rather, suggest that in response to strong signal, T cells or thymocytes may first respond, if contextually necessary, by reducing TCR-directed signaling and then by modifying CD5 expression.

In conclusion, our novel developmental stage-specific system for TCR signaling modification provides experimental evidence of the existence of tuning mechanisms that allow adjustments in T cell responsiveness to environmental cues and that would limit the existence of tuning mechanisms that allow adjustments in T cell responsiveness.

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**Disclosures**

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**References**


