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Selective Expression of the 21-Kilodalton Tyrosine-Phosphorylated Form of TCR ζ Promotes the Emergence of T Cells with Autoreactive Potential

Lisa A. Pitcher,* Meredith A. Mathis,* Srividya Subramanian,* Jennifer A. Young,* Edward K. Wakeland,* Paul E. Love,‡ and Nicolai S. C. van Oers2*†

T cells undergo negative selection in the thymus to eliminate potentially autoreactive cells. The signals generated through the αβ TCR following receptor interactions with peptide/MHC complexes in the thymus control these selection processes. Following receptor ligation, a fraction of the TCR ζ subunit appears as two distinct tyrosine-phosphorylated forms of 21 and 23 kDa (p21 and p23). Previous data have reported elevated levels of p21 in some murine models of autoimmunity. We have examined the contributions of both the p21 and p23 to T cell negative selection in the HY TCR-transgenic system using ITAM-substituted TCR ζ and CD3 ε transgenic mice. Expression of just p21, in the absence of p23, partially impairs negative selection of self-reactive HY-specific T cells. This result in the emergence of potentially autoreactive peripheral T cells and an elevated population of CD11b⁺B220⁺ B cells in the spleen. These data clearly identify a specific and unique role for p21 during negative selection. The Journal of Immunology, 2005, 174: 6071–6079.

The processes of positive and negative selection are shaped by the ability of the αβ TCR to recognize self-peptide/self-MHC molecules expressed in the thymus (1). These developmental decisions are largely determined by the intracellular signals initiated by the multiple ITAM motifs (ITAMs) present in the cytoplasmic tails of the TCR-invariant chains (reviewed in Refs. 2–4). The ITAMs are signaling motifs present in three copies in the TCR ζ subunit and one copy in each of the CD3 γ, δ, and ε subunits (reviewed in Refs. 2–4). Once the TCR engages a self-peptide/self-MHC complex, a subset of the ITAMs becomes transiently phosphorylated on tyrosine residues. This results in the recruitment and activation of the Syk family of protein tyrosine kinases, with each biphosphorylated ITAM associating with one molecule of ZAP-70 (4, 7). The current structural model for the TCR complex provides for a total of 10 ITAMs (αβ TCR with TCR ζζ and CD3 γεδε) (6). These 10 ITAMs can function in an additive capacity during both positive and negative selection (8, 9). For αβ TCR-transgenic mice expressing high-avidity TCRs (P14 and 2C), only a few ITAMs are required for efficient selection (reviewed in Refs. 4, 5, and 10). Upwards of 6–10 ITAMs are necessary for effective positive and negative selection in TCR-transgenic lines with low-avidity TCRs (HY, DO11.10, and AND).

In fact, the loss of two ITAMs in the HY system can reduce the efficiency of both positive and negative selection (9).3 A portion of the 16-kDa TCR ζ subunit (p16) can form two predominant tyrosine-phosphorylated derivatives with distinct molecular masses of 21 kDa (p21) and 23 kDa (p23), respectively (reviewed in Ref. 10). In thymocytes and peripheral T cells, a percentage of TCR ζ exists in a constitutively tyrosine-phosphorylated state, characterized by a specific molecular mass of 21 kDa (p21) (11–15). p21 is formed following the complete phosphorylation of the two membrane-distal ITAMs of TCR ζ and is actually stabilized by complexing an inactive pool of ZAP-70 molecules (12, 13). The function of p21 has been somewhat of a conundrum (16, 17). First, it has been suggested that p21 serves to enhance T cell responses to foreign Ags (18, 19). Second, studies using T cell clones have indicated that p21 can actively inhibit T cell responses to agonist peptides (16). In addition, the preferential expression of p21 in response to antagonist peptides has been either linked or completely unconnected from induction of anergy in T cells (20–23). We have previously demonstrated that the select expression of p21, in the absence of p23, has no discernable negative impact on T cell signal transmission (5).3 In fact, TCR-mediated signaling is completely normal in the absence of all phospho-ζ intermediates.3 The constitutive expression of p21 occurs in both thymocytes and peripheral T cells (11, 13, 19). Interestingly, elevated levels of p21 in the periphery have also been found in autoimmune strains of mice, suggesting a role for p21 in the processes of autoimmunity (24, 25). To more carefully assess the functions of the phosphorylated forms of TCR ζ on negative selection and the role of p21 in autoimmunity, we used a series of TCR ζ transgenic mice in which specific tyrosine residues in the TCR ζ ITAMs were substituted with phenylalanine. All the transgenic lines are on a TCR ζ-null background. In vivo, thymocytes and peripheral T cells from these different mice selectively expressed just p21 in the absence of p23 (YF1,2 line), weakly phosphorylated intermediates of 19- and 20-kDa (YF5,6 line), or no tyrosine-phosphorylated forms of TCR ζ (YF6-1 line) (5, 10, 12). Our analyses also included a CD3 ε ITAM mutant (CD3 EM), which bears tyrosine to phenylalanine substitutions in the CD3 ε ITAM, while retaining all TCR ζ ITAMs and...
phospho-ζ intermediates (26). Notably, the YF1,2, YF5,6, and CD3 e M mutant lines all express 8 of 10 ITAMs within the TCR complex. All of these lines were mated to the HY TCR-transgenic mice that express an αβ TCR specific for the male HY peptide, Smcy. The male HY TCR mice are routinely used as a model system for studying negative selection (26, 27).

In this report, we provide direct evidence that the selective expression of p21, in the absence of p23, modifies negative selection in the HY/YF1,2 male mice, facilitating the development of potentially autoreactive T cells. Thus, the HY/YF1,2 male mice maintain expression of T cells bearing the autoreactive TCR with increased levels of the CD8 coreceptor and higher levels of CD5 compared with the other HY and HY/YF lines. Splenocytes from the HY/YF1,2 male mice also contain an increased percentage of CD4+CD69+ T cells and an expanded population of CD11b+ B220+ B cells in the spleen. These phenotypes are very distinct from those characterized in the HY/YF5,6, HY/YF1-6, and HY/CD3 e M male mice. These results provide the first direct demonstration of important functional distinctions between different ITAMs in the TCR ζ subunit during T cell development, and reveal a link between p21 and autoreactive potential.

**Materials and Methods**

**Abs and peptides**

Biotinylated, FITC-, PE-, allophycocyanin-, and/or CyChrome-conjugated Abs with the following specificities were used: CD1, CD1e, CD4, CD5, CD8α, CD8ß, CD11b, CD19, CD21/35, CD23, CD25, CD43, CD44, CD45RA, CD45RB, CD62L, CD69, CD86, CD94, CD138, IgM, and NK1.1. The Abs were obtained from BD Pharmingen or CalTag. The HY-specific clonotypic mAb (T3.70) detecting the HY TCR ζ transgene (hybridoma generously provided by Dr. H.-S. Teh, University of British Columbia, Vancouver, British Columbia, Canada) was purified with protein A and labeled with fluorescein or biotin. FITC-conjugated goat anti-mouse IgG Abs were purchased from CalTag Laboratories. The HY peptide (Smcy) and control peptide (AV) were previously described (28). Western blotting was undertaken as described using either anti-phospho-tyrosine (4G10; Upstate Biotechnology) or the indicated mAbs followed by a goat anti-mouse IgG (HRP conjugate) from Zymed Laboratories (5).

**Transgenic mice**

TCR ζ-transgenic mice bearing selected tyrosine-to-phenylalanine substitutions in the TCR ζ ITAMs were designated YF1,2, YF5,6, and YF1-6 as described (5, 10, 12). T cells from the YF1,2 line constitutively express p21 and are unable to generate p23. The YF5,6 line expresses weak p19/p20 phospho-ζ intermediates, which are detected after TCR cross-linking (12). The YF1-6 line expresses no phospho-ζ intermediates (10, 12). All of the HY TCR ζ transgenic lines, maintained on a TCR ζ-null background, were backcrossed onto the HY TCR-transgenic line and were designated as HY/YF1,2, HY/YF5,6, and HY/YF1-6.3 Similar patterns of phospho-ζ were detected in the male HY YF-transgenic lines as those seen in wild-type YF and P14/YF mice, as well as the HY/YF TCR-transgenic female mice (5, 10, 12, 29) (data not shown). The CD3 e mutant mice (CD3 eM), lacking two ITAMs because CD3 e is present in two copies in the TCR complex, were also maintained on an HY background (26). For all of the mice analyzed, the axillary, lateral axillary, mesenteric, and superficial inguinal lymph nodes were pooled.

**T cell proliferation assays and CD69 up-regulation**

For most of the experiments outlined, age-matched male mice of 5–10 wk of age were used. Proliferation assays were essentially as described,3 with the exception that exogenous IL-2 (20 U/ml) was added to all cultures. CD69 up-regulation was also described previously,3 with the exception that 1 × 10⁶ total thymocytes from the various HY and HY/YF male mice were used in the analyses. CD69 up-regulation in total thymocyte populations was represented as fold increase in the percentage of cells expressing CD69 following incubation with high concentrations of agonist peptide relative to incubation of whole thymocytes with no peptide controls. The results are representative of at least three independent experiments. For all of the experiments, the double-positive and double-negative populations of thymocytes were also analyzed for CD69 expression by electronic gating for the CD4 and CD8 coreceptor molecules.

**Isolation of intraepithelial lymphocytes (IELs)**

The small intestines of the HY and HY/YF male mice were removed, washed with PBS, and incubated for 30 min at 37°C in a PBS-based extraction solution containing 3% FCS, 1 mM DTT, and 1 mM EDTA. Peyer’s patches were removed before the lymphocyte isolation. The isolated IELs were washed and stained for flow-cytometric analysis as described (30).

**Analysis of aged mice**

Male HY or HY/YF mice were aged to 8–12 mo in the specific pathogen-free colony at University of Texas Southwestern Medical Center, at which time they were sacrificed and bled by cardiac puncture. Sera from the various mice were tested for the presence of IgM and IgG autoantibodies against total histone/dsDNA by ELISA as described previously (31). These mice were compared with B6.Sle1b mice, a congenic strain exhibiting loss of tolerance to nuclear Ags, one of the hallmark features of systemic lupus erythematosus (32).

**Results**

The 21-kDa tyrosine-phosphorylated form of TCR ζ selectively attenuates negative selection in male HY TCR-transgenic mice

The HY TCR-transgenic male mice are routinely used as a model for studying negative selection, in part because the agonist peptide (Smcy) for the HY TCR is naturally expressed in the thymus (27). Previous studies with this model system have shown that the efficiency of negative selection is directly correlated with the number of ITAMs present in the TCR complex (8, 9). Because these studies were undertaken with particular TCR ζ truncations preventing the formation p21 and/or p23, we wanted to carefully examine how p21 and other phospho-ζ intermediates contributed to negative selection (16). For this purpose, we bred the HY TCR-transgenic mice to distinct TCR ζ transgenic lines that selectively expressed only the constitutively phosphorylated 21-kDa form of TCR ζ (p21; YF1,2), two weak inducibly phosphorylated forms of TCR ζ (p19/p20; YF5,6), or no tyrosine-phosphorylated forms of TCR ζ (YF1-6) (5, 10, 12). The YF1,2 and YF5,6 lines contain an equivalent number of ITAMs (8 of 10) in the TCR, while expressing distinct phosphorylated forms of ζ. An independent set of mice with specific mutations in the CD3 e ITAMs (CD3 eM), which contain 8 of 10 ITAMs and express both p21 and p23, were also included in these analyses (26).

In wild-type HY TCR-transgenic male mice, the majority of thymocytes are deleted, resulting in a residual population of CD4+CD8+ thymocytes that represent >75% of total thymocytes (Fig. 1A). Consequently, there is an almost complete absence of mature CD4+CD8+ and CD4+CD8+ T cells in these mice, consistent with that previously published (27, 33, 34). In contrast, the introduction of the YF1,2 ζ transgene into HY male mice resulted in less efficient negative selection, with a statistically significant increase in the percentage and number of CD4+CD8+ thymocytes expressing the transgenic receptor (T3.70) when compared with the HY male mice (p < 0.02; Fig. 1, A and B; Table I). Small numbers of mature CD4+CD8+ and CD4+CD8+ T cells were also observed in the thymus. Interestingly, there was a decrease in the TCR density of the HY/YF1,2 thymocytes (mean fluorescence intensity [MFI] = 57.1) compared with HY male mice (MFI = 122.1). When the CD4+CD8+ and CD4+CD8+ thymocyte populations were analyzed separately, it was determined that the TCR density in the CD4+CD8+ population was substantially decreased. This contrasts the HY male mice, which express high levels of the male-specific TCR in the CD4+CD8+ population (data not available from http://www.jimmunol.org/ by guest on November 7, 2017

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4 Abbreviations used in this paper: IEL, intraepithelial lymphocyte; MFI, mean fluorescence intensity.
Thus, the select expression of p21 in the HY/YF1,2 males results in a distinct down-modulation in TCR expression on the CD4⁺/CD8⁺ thymocytes and reduces negative selection. Because the YF1,2 line has only 8 of 10 ITAMs, the reduced efficiency of negative selection may have been caused by the loss of ITAMs. We next analyzed the HY/YF5,6 line, which maintains

*Statistical values for the percentage and number of thymocytes in the HY and HY/YF male mice were determined from n = 6 (means ± SEM).

Two HY/CD3 eM male mice were analyzed and exhibited similar numbers and percentages of cells.
Peripheral T cell phenotypes are altered by the select expression of p21

To determine how the attenuated negative selection processes in the various HY/YF mice affected peripheral T cell development, we analyzed lymph node T cells. In HY male mice, an unusual expression of the 21-kDa tyrosine-phosphorylated form of TCR \( \zeta \) in HY male mice results in the appearance of potentially autoreactive peripheral T cells. A, Lymph node preparations from HY TCR-transgenic male mice and age-matched HY male mice expressing the YF1,2, YF5,6, or YF1-6 TCR \( \zeta \) constructs on the TCR \( \zeta \)-null background were obtained. A, Single-cell suspensions were prepared and stained with mAbs to CD4 and CD8 and/or T3.70 and analyzed by flow cytometry. B, Histogram analyses for the levels of T3.70 and CD8 expression were compared in the different HY and HY/YF male mice. C, Peripheral CD4\(^+\)T3.70\(^+\) T cells were stained with fluorochrome-labeled mAbs against the cell surface proteins CD44, CD62L, and CD5. The results presented in this figure are representative of six different experiments, with the exception of C, where two independent experiments were analyzed.

an equivalent number of ITAMs (8 of 10) as the HY/YF1,2 line, but expresses distinct phospho-\( \zeta \) intermediates of p19 and p20, forms not normally seen in wild-type mice. In these mice, there was also an increase in the percentage of CD4\(^+\) CD8\(^-\) thymocytes relative to HY male mice (Fig. 1A; Table I). Despite the increase in double-positive thymocytes, the number of CD4\(^+\)CD8\(^-\) and CD4\(^-\)CD8\(^+\) T cells expressing normal coreceptor levels in thymocytes from the HY/YF5,6 male mice was as low as that seen in the HY/YF1,2 male mice. In addition, the thymocytes from the HY/YF5,6 line did not exhibit the reduction in TCR density noted in the CD4\(^+\)CD8\(^-\) population from the HY/YF1,2 line. Taken together, these results strongly support the contention that expression of p21, in the absence of p23, attenuates negative selection. As an extension of these findings, we also analyzed the HY/YF1-6 male mice, which lack all phosphorylated TCR \( \zeta \) intermediates. In these male mice, the reduced number of ITAMs also reduces the efficiency of negative selection, as indicated by the large percentage of CD4\(^+\)CD8\(^-\) thymocytes and high expression levels of the HY-specific TCR (Fig. 1A). In these lines, detectable numbers of mature CD4\(^+\)CD8\(^-\) are evident in the thymus, findings consistent with earlier studies using HY/\( \zeta \)-transgenic lines lacking the cytoplasmic tail of \( \zeta \) (9). The HY/\( \zeta \)\(^{-/-}\) thymocytes were included as controls revealing that the absence of \( \zeta \) expression results in the appearance of a high percentage of CD4\(^+\)CD8\(^-\) thymocytes that lack TCR expression.

To assess whether the impaired negative selection detected in the HY/YF1,2 male mice resulted in alterations in TCR signal strength, the cell surface expression of CD5 and CD69 were compared on both the CD4\(^+\) CD8\(^-\) and CD4\(^-\)CD8\(^+\) thymocytes. Similar levels of CD5 were expressed in the HY, HY/YF1,2, and HY/YF5,6 male mice in both the CD4\(^+\)CD8\(^-\) and CD4\(^-\)CD8\(^+\) thymocyte populations (Fig. 1C). In contrast, the CD4\(^+\)CD8\(^-\) and CD4\(^-\)CD8\(^+\) thymocyte subsets from the HY/YF1-6 male mice expressed diminished levels of CD5 on the cell surface when compared with the other HY and HY/YF lines. This likely occurs as a compensatory mechanism for the decreased TCR signal strength due to the loss of all six TCR \( \zeta \) ITAMs in HY/YF1-6, because a different set of fluorochrome-labeled mAbs was used. Overall, these results suggest that a reduction in the various HY/YF mice affected peripheral T cell development, we analyzed lymph node T cells. In HY male mice, an unusual
lineage of CD4+CD8− and CD4+CD8low− T cells that express the autospecific TCR emerge in the periphery (Fig. 2A) (data not shown) (34). The down-modulation of CD8 on the cell surface renders them unresponsive to the male Ag in vivo (33). These cells represent a unique population of cells, exhibiting a memory-like phenotype with more innate cell properties (35, 36). In contrast to HY male mice, the HY/YF1,2 line contained an increased percentage of mature CD4+CD8− T cells (15.0%) (Fig. 2A; Table II). Unexpectedly, the CD4−CD8+ T cells that emerged in the HY/YF1,2 line maintained expression of the autoreactive TCR, albeit at reduced levels, and contained an intermediate level of CD8 co-receptor (CD8m) when compared with HY male (CD8low) and female mice (CD8high) (Fig. 2B; data not shown). These levels are similar to the HY-specific cells that develop in a partially selecting background (35). The HY/YF5,6 male mice contained an increased percentage of CD4+CD8− (7.9%), similar to the percentages in the HY/YF1,2 line (Fig. 2A; Table II). However, in contrast to the HY/YF1,2 CD4+CD8− T cells, the HY/YF5,6 male mice contained the CD4−CD8+ evident in the wild-type HY males (Fig. 2B). These results reveal a very critical distinction in the ability of p21, in the absence of p23, to facilitate the emergence of T cells that are potentially autoreactive. When the lymph nodes of the HY/YF1-6 male mice were examined, there was an increase in the percentage of CD4+CD8− T cells (14.8%) and a reduction in the percentage of CD4+CD8+ T cells (8.1%) compared with the HY/YF1-6 male mice. These data clearly demonstrate a distinct functional role for p21 in regulating negative selection.

Ag reactivity of HY/TCR ζ-transgenic male thymocytes

We next compared the T cell responses to cognate male Ag in the different HY and HY/YF lines. We initially analyzed the capacity of these cells to express CD69, an early marker of T cell activation. Whole thymocyte preparations from male HY or male HY/YF series mice were cultured for 19 h with APCs presenting either no peptide or increasing concentrations of the agonist peptide Smcy, and subsequently analyzed by flow cytometry. HY-specific T cells from female mice were included for comparative purposes. No response was detected using peptide concentrations of 10−8 M. The percentage of the cells expressing CD69 following incubation with high agonist peptide concentrations (10−4 M Smcy) ranged from 24 to 68% (Fig. 3). The values were somewhat lower with lower peptide concentrations (10−6 M), ranging from 16.8 to 28.1% for the HY, HY/YF1,2, and HY/YF5,6 lines. Surprisingly,

![FIGURE 3. CD69 up-regulation following agonist peptide stimulation is comparable in thymocytes from the various HY and HY/YF male mice. Thymocytes were isolated from the HY or HY/TCR ζ-transgenic male mice and cultured for 19 h at 37°C with APC pulsed with agonist peptide (Smcy) or no peptide. The expression of CD69 on the cell surface was assessed by flow cytometric analysis of cells stained for CD4, CD8, and CD69. CD69 induction in whole thymocyte preparations is represented as fold increase in the percentage of cell expressing CD69 following stimulation with a high dose of agonist peptide (10−3 M) relative to no-peptide control cultures. Results are representative of at least three independent experiments.](image-url)
the percentage of cells expressing CD69 was higher in the HY/YF1-6 lines, even at lower peptide concentrations, followed by the HY/YF5,6, HY/YF1,2, and then the HY male cells. However, the values did not appear to be statistically significant. We also represented the data as a fold-induction in CD69 expression with no peptide vs agonist peptide. The fold increases again ranged from 3- to 15-fold among the different mice, but the differences were not statistically significant. Also, no obvious difference in the percentage of CD69-expressing cells among the CD4^+ CD8^- and CD4^+ CD8^- subsets was uncovered (data not shown). It was also noted that the degree of CD4 and CD8 coreceptor down-modulation in response to agonist peptide stimulation was comparable in the various HY and HY/YF lines (data not shown). In summary, the elevated expression of CD8 in the HY/YF1,2 thymocytes did not correlate with enhanced signaling functions, as measured by the percentage of cells expressing CD69.

**Proliferative responses of the HY TCR \( \xi \) transgensics**

We next evaluated the proliferative capacity of the HY and HY/TCR \( \xi \) transgenic male thymocytes and lymph node T cells in response to Smcy peptide-pulsed APCs. The wild-type HY male thymocytes exhibited a high basal rate of proliferation and responded only marginally, with \(-1.5\) - 2-fold increase in proliferation, with increasing Smcy concentrations (Fig. 4A). When comparing the HY/YF1,2 and HY/YF5,6 lines, a similar 3- to 5-fold increase in dose-responsive proliferation to the agonist peptide was detected (Fig. 4A). The HY/YF1,6 line responded with a 5- to 6-fold increase in proliferation at high doses of agonist peptide (Fig. 4A).

The proliferative responses of mature T3.70^+CD8^+ cells isolated from the lymph nodes were then compared in the various HY/YF male mice and HY female and male mice. In the absence of exogenous IL-2, the HY male cells were significantly impaired in their ability to proliferate to male peptide when compared with HY female cells (data not shown). Only in the presence of IL-2 did the HY male cells proliferate to high doses of agonist peptide (Fig. 4B). When the peripheral T cells from the HY/YF1,2, HY/YF5,6, and HY/YF1-6 male mice were examined, nearly identical dose-response curves were observed (Fig. 4B). All of the HY and HY/YF lines appear less responsive to low agonist peptide doses when compared with HY females (Fig. 4B). We also compared the proliferative responses to the T cell mitogen, Con A. All of the HY and HY/YF male T cells isolated from the lymph nodes proliferated equally to mitogenic stimulation (data not shown). These data indicate that, despite distinct phenotypic differences in the T cells from the HY/YF males, these cells all retain a similar capacity to respond to their cognate male peptide, Smcy, in vitro. This is consistent with our previous findings in the HY/YF female lines.3

**Autoimmune phenotype in aged mice**

Previous studies have drawn correlations between enhanced expression of p21 and autoimmune disease (24, 25). To ascertain whether autoimmunity is induced by the selective expression of p21 in the HY/YF1,2 line, in which less efficient negative selection occurs enabling the generation of a population of potentially autoreactive cells, we analyzed HY vs HY/TCR \( \xi \) transgenic male mice that had been aged 8–12 mo. The various HY and HY/TCR \( \xi \) transgenic mice were compared for signs of autoimmunity, including changes in T and B cell populations, activation markers, and the generation of autoantibodies. In the spleens of the HY/YF1,2 males, an increased percentage of CD11b^-B220^- cells was noted compared with control HY males and from both the HY/YF5,6 and HY/YF1-6 lines (\( p < 0.01 \)) (Fig. 5A). These cells were analyzed further, and it was determined that the CD11b^-B220^- cells expressed CD19, indicating that they represent a subset of B cells (data not shown). When we assayed the sera in the various HY and HY/YF male mice for the presence of Abs against total histone/dsDNA, as an indication of systemic autoimmunity, the HY/YF1,2 male mice exhibited only a slight increase in the presence of autoantibodies compared with the HY, HY/YF5,6, and HY/YF1-6 male mice. However, these values were not statistically significant (Fig. 5B). The levels of autoantibody present in the HY/YF1,2 males were below the threshold considered to be autoimmune (>200 arbitrary units), which is represented by the B6.Sle1b mice (32). Although the HY/YF1,2 male mice did not exhibit severe autoimmunity by 8–12 mo of age, these mice did demonstrate an increase in T cell activation phenotypes relative to the HY, HY/YF5,6, and HY/YF1-6 male mice. Variations in the activation state of the CD4^+ T cells were noted, particularly the aged HY/YF1,2 male mice. A significant increase in the percentage of CD4^- T cells expressing the early activation marker CD69 was detected in the HY/YF1,2 mice when compared with all other groups (\( p < 0.01 \)) (Fig. 5C). In addition, the percentage of CD4^- T cells expressing CD25 was significantly reduced in the HY/YF1,2 line when compared with the HY males (\( p < 0.05; \) Fig. 5C). Surprisingly, there were no statistically significant differences in the activation state of the CD8^- T cells in the spleens of the aged HY/YF1,2 male relative to the wild-type HY male mice, although similar trends were noted (Fig. 5D). Overall, the increase in activated CD4^- T cells and CD11b^-B220^- B cells in the HY/YF1,2 male mice may be indicative of a higher potential for autoimmune disease that may require a triggering event such as infection.
IELs are not altered in the p21-expressing HY male mice

It has previously been reported that self-reactivity in thymic CD4+CD8+ cells can result in the development of CD8α T cells with innate immune cell characteristics (35, 36). Because the HY/YF1,2 male mice develop T cells with autoreactive potential that arise from thymocytes, we wanted to assess whether these male mice had increased representation of IELs expressing the CD8α coreceptor pair. To examine this possibility, we isolated IELs from the various mice and stained the lymphocytes with mAbs against CD8α and CD8β. We determined that the percentage and number of CD8α and CD8αβ T cells were similar among the various mice (Fig. 6).

Discussion

We have examined the contributions of different ITAMs (TCR and CD3) in the process of negative selection in the thymus. In particular, we focused on the role of the constitutively tyrosine-phosphorylated TCR subunit (p21), because its expression has been linked to autoimmune progression. We demonstrate herein that the expression of p21, in the absence of p23 (HY/YF1,2 line), promotes the development of potentially autoreactive T cells in the HY TCR-transgenic male mice. Three unique features of the HY/YF1,2 line included the emergence of a population of peripheral T cells expressing the autoreactive TCR and increased levels of CD8 on the cell surface, increased percentages of activated CD4+ T cells, and the expansion of B1 B cells in the spleen. In contrast, the HY/YF5,6 and HY/CD3M lines, both of which contain the same number of TCR ITAMs as the HY/YF1,2 line, had mature T3.70CD8low T cells that resembled those from the HY male.

Most studies to date have revealed an additive effect for the ITAMs during thymopoiesis, wherein a reduction in the number of TCR ITAMs reduces the efficiency of positive and negative selection (8, 9). This was consistently observed in transgenic lines bearing low-avidity TCRs. However, no experiments were undertaken to address the specific functions of p21. Our previous studies, in which we examined the contributions of phospho-ζ to T cell-positive selection, are consistent with the notion that the TCR ζ and CD3 ITAMs function additively, in that sets of mice lacking 2 of 10 ITAMs in the TCR complex (YF1,2; YF5,6) had similar reductions in the efficiency of positive selection. Somewhat surprisingly, the constitutive expression of p21 (YF1,2) offered no obvious selection advantage to T cells. Our current studies indicate that during negative selection, the ITAMs can also function in an additive manner, because the HY/YF1,2, HY/YF5,6, and HY/CD3M lines (all containing 8 of 10 TCR ITAMs) all show increased...
percents of CD4+CD8+ thymocytes. In fact, the inefficient negative selection in the HY/YF1-6 male mice seems to facilitate gene rearrangements of endogenous TCR α-chains, as evidenced by the emergence of “normal” peripheral CD4+CD8− T cells. However, the results described in this report indicate a unique function for p21 in negative selection, resulting in the emergence of a CD8silencedT3.70+ population of T cells not previously identified in HY male mice nor in the HY/YF5.6, HY/YF1-6, or HY/CD3 eM male mice. This is the first demonstration that the different phosphorylated ITAMs can contribute differentially during negative selection.

How does the expression of p21, in the absence of p23, alter negative selection? One possibility is that the selective expression of p21 alters the functional capacity or spatial organization of signaling molecules required for negative selection. It has been established that p21 associates with an inactive pool of ZAP-70 (13). During negative-selection events, the localization of signaling molecules, including Lck and TCR ζ, are distinct from that seen during positive selection or even mature T cell activation (37). Specifically, Lck is localized to the center of the immunological synapse, whereas TCR ζ appears in a peripheral ring. Given the association of ZAP-70 with p21, ZAP-70 would also be localized at the periphery. If p21 sequesters ZAP-70 in the peripheral ring of the synapse, the activation of ZAP-70 by Lck might be ineffective in p21-expressing thymocytes. In wild-type HY or HY/CD3 eM male mice, TCR-mediated induction of p23, the fully phosphorylated form of TCR ζ, could override this block. p23 might efficiently recruit new molecules of ZAP-70, allowing for their proper localization and activation. Alternatively, the p21/ZAP-70 complex may include attenuators of signal transduction such as cbl-b and/or Sigs proteins, both of which can bind ZAP-70 (38, 39). In the wild-type HY and HY/CD3 eM males, the induced expression of p23 could displace these signal inhibitors. In the absence of p23 in the HY/YF1,2 line, the failure to recruit sufficient forms of activated ZAP-70 might prevent efficient removal of these signal attenuators. When examined during positive selection events in the female HY/YF mice, the expression of p21, in the absence of p23, had no discernable effects on ZAP-70 activation or positive selection. This could result from a different spatial organization of the signaling molecules that occurs during positive-relative to negative-selection events (37). In the absence of both p21 and p23 in the HY/YF5.6 and HY/YF1-6 lines, negative selection could be restored by activation signals mediated via the CD3 ηε/δε signaling module. These lines may have an inefficient negative selection, facilitating the development of CD4+CD8+ cells.

It has also been reported that mutations in key signaling molecules, including ZAP-70, can diminish the intensity of proximal TCR signaling events, leading to impaired thymic selection and the emergence of spontaneous autoimmunity (40). Thus, the sequestration of ZAP-70 by p21 in the HY/YF1,2 line could function in a similar manner, diminishing the intensity of signals in situ that are required for negative selection. Interestingly, in our in vitro assays, the signaling capacity of the thymocytes and peripheral T cells from the HY and HY/YF lines are equivalent. Additional experiments are being undertaken to explore these possibilities, but suggest that the CD3 ηε/δε subunits form the predominant signaling module, as ascertained by in vitro assays.

Previous reports have identified a distinct phenotype for the cells that escape negative selection in the HY system, resulting in a change from CD8αβ to CD8αα cells, which more closely resemble the IELs found in the gut (36). These cells also have acquired characteristics of innate immune cells through the expression of certain activating NK receptors (NK1.1, CD94, and NKG2D) and NK cell-specific signaling molecules (DAP12) (35, 36). In contrast to these studies, we did not detect an increased percentage of CD8αα IELs, nor did we detect enhanced innate cell markers, enhanced production of IFN-γ, or proliferation to IL-2 and IL-15 in the various HY/YF lines, even when one or more phosphorylated TCR ζ intermediates were eliminated (Fig. 6; data not shown). Furthermore, when the activation state of the T3.70+CD8+ cells was analyzed in the HY/YF1,2 male mice, the expression patterns of CD25, CD69, and CD44 were similar to that observed in the HY male mice (data not shown). Although there were no gross autoimmune phenotypes in these mice, these HY/YF1,2 cells may still be capable of generating autoimmunity following some initial triggering event, such as infection. In fact, aged HY/YF1,2 male mice, we observed slightly elevated levels of autoantibodies and increased numbers of CD11b+B220+ B cells only in the HY/YF1,2 line. These markers are present on B1 B cells, which are generally involved in natural Ab production and function in an innate capacity (41). This increase in this subset of B cells may be indicative of a potentially autoimmune phenotype, because an increase in B1 B cells has been observed in murine models of SLE (42). The increased presence of CD11b+B220+ B cells may be an indirect result of the select expression of p21, in the absence of p23, which may change the cytokine milieu to support the expansion of these cell types. In addition, during the aging process required for these studies, the deaths of two HY/YF1,2 male mice were noted. Without pathological evidence, it can only be speculated that the specific increase in mortality in the HY/YF1,2 was a result of autoimmunity. It is also unclear whether the HY-specific T cells are contributing to the pathogenesis, or whether a combination of HY-specific and endogenous TCR α-expressing T cells are necessary for disease progression.

In summary, the TCR ζ ITAMs contribute both additive and distinct functions during thymocyte negative selection, with the select expression of p21 attenuating negative selection. This is the first demonstration that the different phosphorylated ITAMs have distinct functions and are simply not involved in an additive capacity. Current efforts are addressing the unique function of p21 during negative selection events.

Disclosures
The authors have no financial conflict of interest.

References


